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Chromophore formation and phosphorylation analysis of constitutively active rhodopsin mutants

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G protein coupled receptors (GPCRs) transmit various extracellular signals into the cells. Upon binding of the ligands, conformational changes in the extracellular and/or transmembrane (TM) domains of GPCRs were propagated into the cytoplasmic (CP) domain of the molecule leading to the activation of their cognate heterotrimeric G proteins and kinases. Constitutively active GPCR mutants causing the activation of G Protein signaling even in the absence of ligand binding are of interest for the study of activation mechanism of GPCRs. Two classes of constitutively active mutations, categorized by their effects on the salt bridge between E113 and K296, were found in the TM domain of rhodopsin. Opsin mutants containing combinations of the mutations were constructed to study the conformational changes required for the activation of rhodopsin. Rhodopsin chromophore regenerated with 11-*cis*-retinal showed a thermal stability inversely correlated with its constitutive activity. In contrast, rhodopsin mutants exhibited a binding affinity to an agonist, all-*trans*-retinal, in a constitutive activity-dependent manner. In order to test whether the conformational changes responsible for the activation of transducin (Gt) are the same as the conformation required for the recognition of rhodopsin kinase, analysis of the mutants were carried out with phosphorylation by rhodopsin kinase. Rhodopsin mutants containing combinations of different classes of the mutations showed a strong synergistic effect on the phosphorylation of the mutants in the dark as similar to that of Gt activation. The results suggest that at least two or three kinds of segmental and independent conformational changes are required for the activation of rhodopsin and the conformational changes responsible for activating rhodopsin kinase and Gt are similar to each other.

Key words – G protein-coupled receptor, signal transduction, constitutive activation, rhodopsin kinase

Introduction

G protein coupled receptors (GPCRs) represent one of the largest membrane receptor families and play an important role in transmitting various extracellular signals such as hormones, neurotransmitters, and physiological (olfactory, taste and visual) stimuli into inside the cells [9,15]. While GPCRs recognize a variety of signals, all the GPCRs share a common structural topology, seven transmembrane helices, suggesting a common activation mechanism in GPCRs [6,8]. Upon stimulation by their ligands, GPCRs initiate two signal transduction pathways leading to the sensitization and desensitization. The former was triggered by the activation of heterotrimeric GTP/GDP-binding proteins which in turn activate or inhibit the secondary effectors leading to the sensitization of the stimuli. The latter was initiated by the activation of their cognate kinases which phosphorylate the activated receptors followed by the binding of the other proteins such as arrestin.

Rhodopsin, the vertebrate dim-light photoreceptor, consisting of an apoprotein, opsin, and 11-*cis*-retinal chromophore, has been employed as a model system for the structural and functional study of GPCRs [10]. Conformational changes in rhodopsin were triggered by the photoisomerization of 11-*cis*-retinal of rhodopsin into all-*trans*- retinal. The resulting structural perturbation in the TM domain was then propagated into the cytoplasmic loop region of rhodopsin where the interaction between rhodopsin and transducin (Gt) and rhodopsin kinase (RK) occurs. There have been indications that one of the photointermediates, metarhodopsin II, is the species responsible for the activation of the both proteins, Gt and RK [1,2,14].

Conformation changes of GPCRs induced by the recognition of their agonists initiate the signal transduction by activating heterotrimeric G proteins. In contrast to the relatively more structural information on the basal state GPCRs largely deduced from the crystal structure of rhodopsin [19,28], only a limited amount of information is available for the activated state conformation of GPCRs

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[6,16]. The phenomena of constitutive activation, leading to the activation of G protein without ligand binding or in case of rhodopsin without light activation, have been found in many GPCRs. Assuming that constitutively active mutants adopt a conformation mimicking the activated state of GPCRs, structural studies on the mutants may provide more detailed information for the activation mechanism of GPCRs. Constitutively active mutant GPCRs are also of interest for their association with human diseases [21].

In rhodopsin, two classes of constitutively active mutations depending on its effect on the salt bridge between E113 and K296 have been found mostly in the TM domain [22,25]. One class includes mutations at positions G90, E113, A292 and K296 with a salt bridge perturbation. The other includes mutations at E134 and M257 positions which seems to be distant from directly affecting the salt bridge. Previous analysis of the mutant rhodopsin using EPR and Gt activation [11,12] indicated that a 'partially' activated state of conformation was adopted in the mutants due to a perturbation in the TM domain. To study the activation mechanism of GPCRs and to determine whether the conformational changes in the mutants are the same, opsin mutants containing combinations of single point mutations G90D, E113Q, E134Q, and M257Y were constructed. The detailed aspects of activation process of rhodopsin were further analyzed by binding of an agonist and an inverse agonist in this study. Constitutive activity analysis of the mutants using phosphorylation assay was carried out to determine whether the conformational changes responsible for Gt activation are the same as the conformational changes required for activating RK. Two general phenotypes, one showing a relatively minor, at most additive effect, and the other showing a strong synergistic effect on constitutive activity were observed depending upon combinations of mutations from the same or different classes, respectively. This provides further evidence for at least two or three kinds of segmental and independent conformational changes occurred during the activation of rhodopsin. The similar effects of the combinations on both Gt and RK activations also support the notion that the conformational changes in the TM domain required for the activation of Gt are similar to that required for the activation of RK.

Materials and Methods

Materials

Adenosine 5′-γ[-^{32}P]-triphosphate (1250 Ci/mmole) was obtained from Dupont/NEN. The nitrocellulose membranes were obtained from Schlecher & Schnell (Keene, NH). Dodecyl maltoside (DM) was obtained from Anatrace (Maumee, OH). Anti rhodopsin mAb, rho-1D4 [17] was purified from a myeloma cell line provided by R. S. Molday (University of British Columbia) and was coupled to cyanogen bromide-activated SepharoseTM4B. Frozen bovine retinae were from J. A. Lawson Co. (Lincoln, NE) and rod outer segment were prepared by the method of Papermaster [20]. 11-*cis*-retinal was prepared from all-*trans*-retinal from HPLC based on the procedure [13] using 6% ethyl ether in hexane as a solvent.

Cloning, expression and purification of rhodopsin mutants

Plasmids containing the mutations G90D, E113Q, M257Y or A292E in the opsin gene were cloned into pMT4 as described [4,5,7,11]. Plasmids containing double or triple mutations in the opsin gene were constructed by combinations of restriction fragments containing each mutation as described [12]. Plasmids were prepared by alkaline lysis method [27]. Wild type and mutant opsin genes were expressed transiently in Cos-1 cells as described [18]. Cells were harvested 50 hr after transfection and were incubated with 10 μM 11-*cis*-retinal or 20 μM all-*trans*-retinal at 4°C for 3 hr with agitation. Mutant rhodopsin was solubilized by addition of 1% DM and purified by using 1D4 Sepharose 4B affinity chromatography as described [24]. Rhodopsin was eluted in buffer containing 2 mM sodium phosphate (pH 6.0), 0.05% DM and 100 μM C'1-9 peptide. Purified rhodopsin was divided into a small volume and, after quick freezing of the samples in liquid nitrogen, stored at -80°C until its further use. The UV/visible spectra of purified mutants were taken with a Perkin-Elmer λ-6 UV/Vis spectrophotometer. For the measurement of the chromophore stability, purified mutant rhodopsin was incubated at 55°C. The decay of the chromophore was measured directly by UV/Vis spectrophotometer in the case of mutants containing E134Q and M257Y upon incubation at the designated temperatures. Chromophore decay in the mutants containing E113Q mutation was carried out after acid treatment.

Preparation of Rhodopsin Kinase

Rhodopsin kinase was prepared from rod outer segment of bovine retina using Heparin sepharose column chromatography [29]. Briefly, RK was extracted from ROS in the

buffer containing 10 mM BTP (pH 8.3), 240 mM KCl, 1 mM EDTA, 0.25% Tween 80, 8 mg/ml soybean phosphatidyl choline, 10 mM hydroxylamine and 1 mM benzamidine under the illumination. After dialyzing the extracts against 1 L of 10 mM BTP (pH 7.5) and 0.4% Tween 80 followed by loading into the heparin sepharose column, RK was eluted in the buffer containing 10 mM BTP (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 0.05% DM and 0.2 mM ATP. The presence of the kinase during and after the purification was analyzed by immunoblotting assays using 6D8 antibody [3]. The amount of the purified RK was determined by micro-Bradford assay as well as activity assay using urea stripped ROS membrane as described [29].

For phosphorylation assay, the reaction mixture contains 120 μl of 10 mM BTP (pH 7.5), 2 mM $MgCl_2$, 100 μM γ-^{32}P-ATP (specific activity ~2,000 cpm/pmole) including the rhodopsin kinase fraction collected. After the addition of 80 μl of purified rhodopsin (0.1 μM) in the dark, the reaction was incubated at 20°C and the aliquots were taken at various time points. The reaction was terminated by the addition of 180 μl of a solution containing 0.8 M KH_2PO_4, 20 mM ATP and 20 mM EDTA. Aliquots were applied to nitrocellulose membrane presoaked in 1 M KH_2PO_4 and 20 mM ATP. After washing the wells once with 250 μl of 1 M KH_2PO_4, nitrocellulose membrane was washed three times with 50 ml of 1M KH_2PO_4. Radioactivity was measured by Cerenkov counting.

Results and Discussion

Chromophore formation in the presence of 11-cis-retinal

Constructions of the opsin mutants containing double or triple combinations of G90D, E113Q, E134Q, and M257Y (Fig. 1) were carried out as described [12]. Upon transient transfection into Cos-1 cells, most of opsin mutants were expressed to a level comparable to that of wild type opsin (data not shown). Rhodopsin chromophore regenerated with 11-*cis*-retinal was purified by 1D4 sepharose column chromatography as described [24]. Previous analysis on the mutant rhodopsin reconstituted with 11-*cis*-retinal [4,5,7] indicated a wild type-like chromophore (absorption maxima~500 nm) formation in the mutants E134Q and M257Y. Rhodopsin mutants G90D and E113Q showed chromophore formation with a slightly altered spectral properties as described [23,26]. Rhodopsin mutants containing double

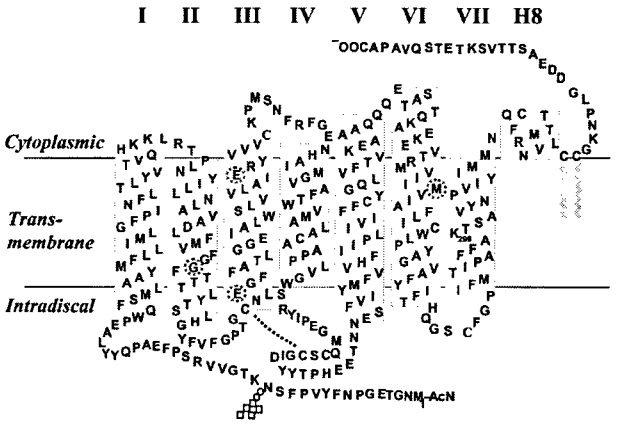

Fig. 1. A secondary structure model of bovine rhodopsin shows the sites of point mutations (marked as squares) examined in this study.

or triple mutations exhibited the spectral characteristics of its composing mutation (Fig. 2) although the level of the chromophore in some mutants is a slightly lower than that of single mutation. This is shown by mutants E113Q/M257Y, E113Q/E134Q, and E113Q/E134Q/M257Y forming rhodopsin chromophore with absorption maxima around 380 nm as consistent with E113Q mutation. While E134Q/M257Y formed a chromophore with an absorption maximum

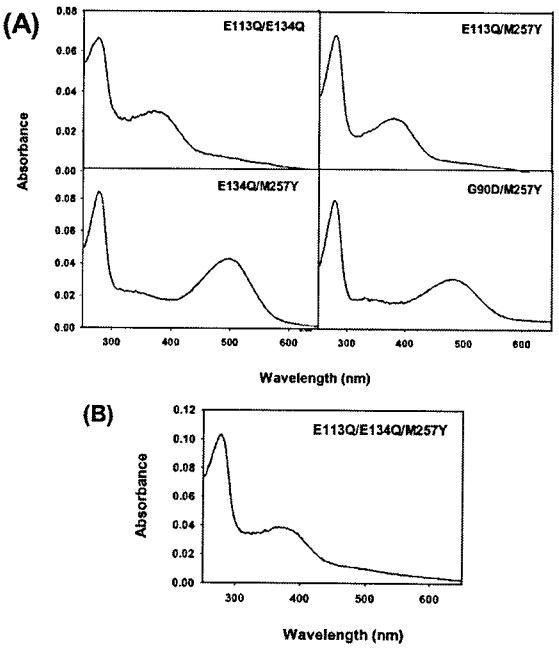

Fig. 2. UV/Vis spectra of rhodopsin mutants containing double (A) and triple (B) mutations. Rhodopsin was purified by 1D4 sepharose chromatography after reconstitution with 10 μM of 11-*cis*-retinal. Rhodopsin mutants eluted in 2 mM sodium phosphate (pH 6.0) and 0.1% DM were subjected to UV/Vis spectroscopy in the dark.

of 500 nm, G90D/M257Y formed a rhodopsin chromophore similar to that of G90D [23].

Thermal stability of rhodopsin mutants reconstituted with 11-cis-retinal

Thermal stability of the mutant rhodopsin was analyzed by the level of chromophore remained upon incubation at the designated temperatures. Rhodopsin mutant E134Q/M257Y was quite stable at temperature 20°C and 37°C as in the case of wild type rhodopsin and single mutation E134Q and M257Y (data not shown). However, all the mutants showed a time-dependent decay of the chromophore at a higher temperature (Fig. 3). In contrast to the relatively slower rate of chromophore decay observed in wild type rhodopsin at 55°C, mutants E134Q and M257Y showed a faster rate of chromophore decay. Rhodopsin mutant E134Q/M257Y exhibited a thermal stability lower than those of single mutants (Fig. 3A). For the mutants containing double or triple mutations including E113Q, the stability of the chromophore was measured by the formation of 440 nm species upon acid treatments as the absorption maxima of 380 nm was overlapped with that of the retinal. All the rhodopsin mutants containing E113Q mutation showed a lower stability than wild type and E134Q and M257Y mutants as reflected by its rapid decay upon incubation at 37°C (Fig. 3B). The result indicates that the E113Q mutation affects more strongly to the stability of chromophore regenerated with 11-*cis*-retinal as compare to those of E134Q and M257Y mutations. The faster decay of the chromophore in the mutants with a higher constitutive activity indicated an inverse relationship between the stability of chromophore regenerated with *11-cis*-retinal and its constitutive activity (see below). Mutants containing G90D showed a higher rate of thermal unstability as in the case of G90D single mutation (data not shown).

Rhodopsin chromophore formation in the presence of all-trans-retinal

To examine the agonist binding affinity of mutant opsins, rhodopsin was reconstituted in the presence of all-*trans*-retinal. Spectral analysis of purified chromophore indicated that all the rhodopsin mutants containing a single mutation as well as wild type rhodopsin showed a low (less than 5% comparing to the chromophore formed in the presence of 11-*cis*-retinal) level of chromophore formation with absorption maxima around 380 nm (Fig. 4). In contrast,

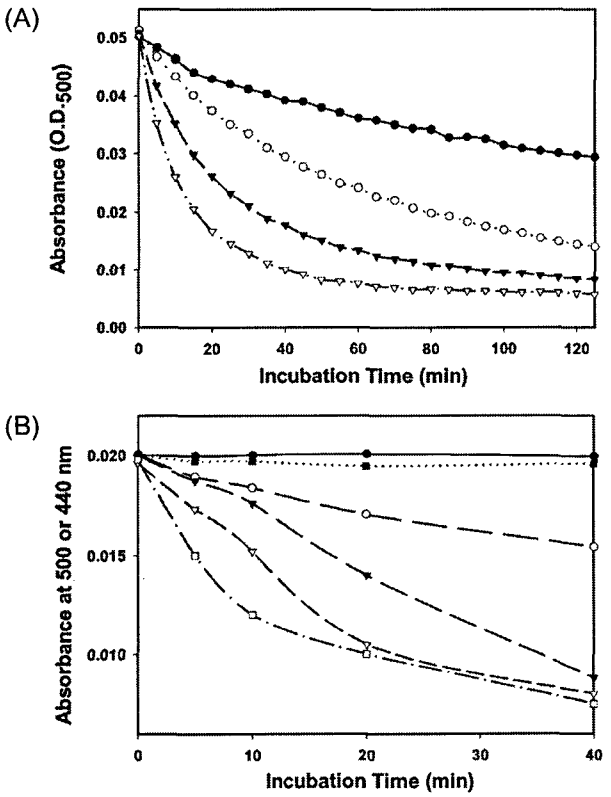

Fig. 3. Thermal decay of rhodopsin chromophore regenerated with 11-*cis*-retinal. (A) Chromophore decay in wild type rhodopsin (closed circle) and the mutants E134Q (open circle), M257Y (closed triangle) and E134Q/M257Y (open triangle) were measured by the extents of 500 nm chromophore remained upon incubation at 55°C. (B) Decay of rhodopsin chromophore with an absorption maxima of 380 nm were measured by the level of 440 nm species appeared upon acidification. Tested were wild type rhodopsin (closed circle), E134Q/M257Y (closed square), E113Q (open circle), E113Q/E134Q (closed triangle), E113Q/M257Y (open triangle), and E113Q/E134Q/M257Y (open square).

rhodopsin mutants E113Q/E134Q and E113Q/M257Y formed a chromophore to the level similar to that of chromophore regenerated with 11-*cis*-retinal. In particular, rhodopsin mutant E134Q/M257Y formed a chromophore with absorption maxima around 380 nm in the presence of all-*trans*-retinal although the same mutant formed a wild type-like (500nm, Fig. 2A) chromophore in the presence of 11-*cis*-retinal. The shift of absorption maxima from 380 nm to 440 nm upon treatment with acids (dotted line) indicated the Schiff's base formation between all-*trans*-retinal and opsin. In addition, rhodopsin chromophore regenerated with all-*trans*-retinal was more stable in the mutant with a higher constitutive activity (data not shown).

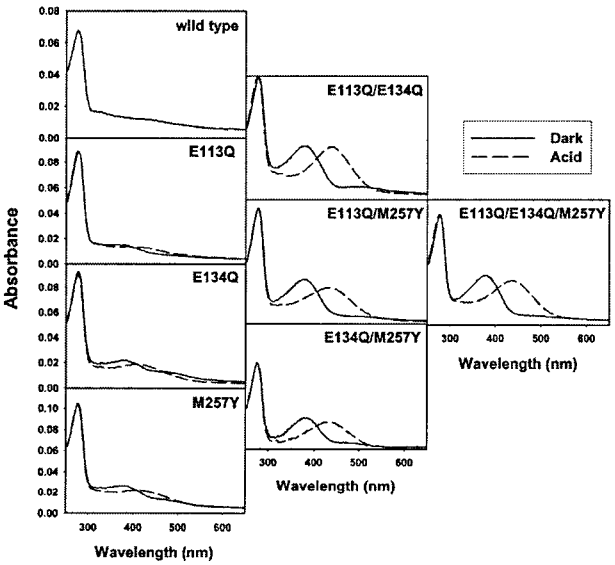

Fig. 4. UV/Vis spectra of rhodopsin mutants reconstitute with all-*trans*-retinal Rhodopsin reconstituted with 20 μM all-*trans*-retinal were purified by 1D4 sepharose chromatography. Rhodopsin were eluted in 2 mM sodium phosphate (pH 6.0) and 0.1% DM. UV/Vis spectra were taken in the in the dark (solid line) and after acidification (broken line).

Activation of rhodopsin kinase by mutant rhodopsin

Differences in the conformational changes occurred in the mutants can be examined by the its level of the constitutive activity. Previous analysis of the mutant rhodopsin using Gt activation in the dark [7,12] indicated that rhodopsin mutants with a single point mutation may adopt only a limited conformational change required for Gt activation as reflected by a low level of constitutive activity. In particular, combination of mutations from different classes resulted in a rather drastic conformational change close to that of the activated state. To further confirm the conformational differences amongst mutants and to test whether the conformational changes responsible for the activation of Gt leading to the sensitization are the same as that required for the desensitization, the effect of combination was analyzed by its ability to be phosphorylated by rhodopsin kinase. For this, rhodopsin mutants were incubated with rhodopsin kinase in the dark and their phosphorylation were measured by the incorporation of ^{32}P using a filter binding assay. For comparison, the level of dark activity exerted by wild type rhodopsin was set as 1 and the activity after the light activation was set as 100, respectively (Fig. 5 & Table 1). Rhodopsin mutants containing single mutations regenerated with 11-*cis*-retinal

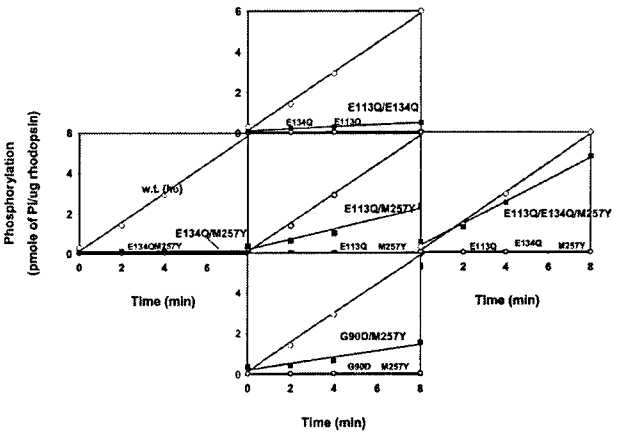

Fig. 5. Comparison of initial rates of phosphorylation of mutant rhodopsin using filter binding assay. Reaction was carried out in 10 mM BTP (pH 7.5), 2 mM $MgCl_2$, 100 μM γ-^{32}P-ATP (specific activity ~2,000 cpm /pmole) and RK. After the addition of purified rhodopsin (0.1 μM) in the dark, aliquots taken at various time points were subjected to filter binding assay. Initial rate of ^{32}P-incorporation in the mutants was compared to those of their constituent mutation as well as light-activated wild type rhodopsin (open circle).

Table 1. Activity analysis of representative rhodopsin mutant

Rhodopsin Mutants	Phosphorylation by RK*	
	Dark	Light
Wild type	1	100
G90D	1.2±0.2	50±10
E113Q	1.6±0.2	1025
E134Q	1.3±0.2	105±5
M257Y	1.4±0.2	110±5
G90D/E134Q	5±0.5	105±10
G90D/M257Y	25±5	105±10
E113Q/M257Y	40±5	120±5
E134Q/M257Y	2.5±0.5	115±5
E113Q/E134Q	7.5±0.5	105±5
E113Q/E134Q/M257Y	80±10	110±5

*Activity comparison was based on the total protein amounts as measured by absorbance at 280nm.

showed only a low level of dark activity. Upon photoactivation, all the single mutants showed light-dependent phosphorylation by RK to a level comparable to that of wild type rhodopsin.

Rhodopsin mutant E134Q/M257Y containing double mutations distantly related to the salt bridge perturbation showed only a moderate (~2.5% of the activity exerted by light-activated wild type rhodopsin) increase in the level of phosphorylation in the dark (Fig. 5). This is at most an additive effect between two mutations, E134Q and M257Y as

compared to that of single mutation (Table1). In contrast, rhodopsin mutants containing double mutations of different classes such as E113Q/E134Q and G90D/E134Q showed a dark phosphorylation activity 4-6 folds higher than the sum of the activity from each mutation. The level of synergistic effects became much stronger when one of the salt bridge-perturbed mutations was combined with M257Y. This was clearly evident in the mutant E113Q/M257Y showing a level of phosphorylation approaching to 40% of the activity exerted by the light-activated rhodopsin. A strong synergistic effect on the constitutive activity was also observed in the mutant G90D/M257Y containing a similar combinations of mutations. The results suggest that the conformational changes occurred in the mutants with perturbed salt bridge (E113Q or G90D) may be different from that of E134Q and, to a larger extent, that of M257Y. Previous analysis of the mutants using Gt activation in the dark also indicated weak additive effects on constitutive activity upon combination of the mutations from the same class [7,12] and a strong synergistic effect on Gt activation upon combination of two classes of mutations. Structural evidence for the conformational changes obtained from EPR analysis [11] showed the activated state-like conformational changes around helix III and helix VI in the mutants E134Q and M257Y, respectively. In addition, rather large conformational changes were detected in all the rhodopsin mutants with perturbed salt bridges [12]. These results are consistent with the notion suggesting that the mutants of the same class adopt only a partially activated conformation that may be similar or partially overlapped each other. Hence, the combination of the double mutations from the same class still generates only a limited conformational change resulting in a low level of constitutive activity while the combinations of mutations from two classes resulted in a synergistic effect on constitutive activity.

Further evidence for the differences in the conformational changes was provided by the phosphorylation level of the triple mutant E113Q/E134Q/M257Y as compared to that of its constituent single or double mutants. If the conformational change in the mutant E113Q, E134Q, or M257Y is the same or partly overlapped to the conformational change in the mutants containing the other two mutations, there may be a little change on constitutive activity upon combinations of the third mutation. In comparison to the level of constitutive activity exerted by the mutants containing double mutations E134Q/M257Y, E113Q/E134Q, and E113Q/M257Y, the triple mutant showed a further synergistic effect on phosphorylation activity upon addition of the third mutation such as E113Q, M257Y, or E134Q, respectively (Table 1). The result provided a further supporting evidence for an independent conformational change adopted in each mutant. The level of constitutive activity in E113Q/E134Q/M257Y is the highest among the mutants and nearly equivalent to that of photoactivated wild type rhodopsin. This indicated that the conformation changes required for full activation of RK may be achieved by combination of the all the changes from each mutation.

In summary, constitutively active rhodopsin mutants were analyzed by their binding to agonist/inverse agonist and by their ability to be phosphorylated by rhodopsin kinase in the dark. While rhodopsin mutants with a stronger constitutive activity showed a higher binding affinity with an agonist in an activity dependent manner, an inverse relationship was found between constitutive activities and binding with an inverse agonist. Rhodopsin mutants with a single mutation showed only a little phosphorylation in the dark. While rhodopsin mutants containing double mutations from the same class showed only a little increase in constitutive activity upon combination, other mutants containing combinations of two class mutations showed synergistic effects on phosphorylation. The relative level of phosphorylation activity amongst double mutants are in the order of E113Q/M257Y > G90D/M257Y > E113Q/E134Q, G90D/E134Q > E134Q/M257Y. The results are consistent with their activity analysis using Gt activation suggesting that the conformational change in the TM domain of rhodopsin responsible for the activation of Gt is similar to that of conformational changes required for RK activation. Rhodopsin mutant E113Q/E134Q/M257Y exerting the highest constitutive activity amongst all known rhodopsin mutants may provide a good model system for obtaining the crystal structure of the activated state of rhodopsin.

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초록 : Chromophore 형성과 rhodopsin kinase 활성을 이용한 항활성 로돕신 mutant의 분석

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G protein-coupled receptor (GPCR)는 세포외부의 신호를 인식 시 G 단백질을 활성화시켜 신호를 전달하며 kinase에 의한 인산화를 통하여 지속적인 신호전달을 억제한다. 외부 신호물질이 없는 조건에서도 활성을 나타내는 항활성 돌연변이종(CAM)은 GPCR의 신호전달 이상에 기인한 질병 치료나 활성화 구조변화의 좋은 연구대상이다. 희미한 빛을 인식하는 시각수용체인 로돕신의 CAM으로는 salt bridge에 직접적인 영향을 미치는 돌연변이인 G90D, E113Q, 그리고 K296E와, 직접적인 영향이 없는 돌연변이인 E134Q 와 M257Y 등 두 가지 계통의 종류가 알려져 있다. 본 연구에서는 각각의 돌연변이가 복합된 mutant를 구성하여 agonist와 inverse agonist에 대한 친화도와 로돕신 kinase에 대한 활성을 조사하여 각 종에서의 구조변화의 차이를 분석하였다. 로돕신 mutant의 constitutive activity는 all-*trans*-retinal에 대한 친화도에 비례하며 11-*cis*-retinal에 대한 친화도와는 역상관 관계를 보여준다. 같은 계통에 속하는 돌연변이가 합쳐진 복합 mutant는 단일 mutant에 비하여 미약한 정도의 로돕신 kinase 항활성화 증가를 보여주나, 다른 계통에 속하는 두 가지 돌연변이가 합쳐진 mutant는 항활성화가 크게 증가되었음을 보여주었다. 이 결과는 다른 계통에 속하는 mutant에서는 상이한 구조변화가 일어나며 로돕신이 완전한 활성화에 이르기 위해서는 최소한 두 가지 종류의 돌연변이에 의하여 생기는 구조변화들이 함께 일어나야함을 의미한다. G protein 활성화와 유사한 항활성화 분석결과는 rhodopsin kinase가 인식하는 로돕신의 활성화상태 구조가 G protein이 인식하는 구조와 유사함을 의미한다. 특히 가장 강한 활성을 나타내는 E113Q/E134Q/M257Y는 활성화상태 GPCR 단백질의 결정 시도에 이용 될 수 있을 것이다.