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論文内容の要旨

G-protein coupled receptors (GPCRs) mediate various physiological responses in cells of organisms. They are expressed on the cell membrane and are known to have extracellular N- and intracellular C-termini, and seven-transmembrane domain (7TMD) as a commonly shared motif. These GPCRs can be categorized into three groups, family A, B, and C. Family A includes rhodopsin and muscarinic acetylcholine receptors (mAChRs), etc. Family B members are basically hormone receptors. The last group, family C, is represented by metabotropic glutamate receptors (mGluRs) and $GABA_B$ receptor ($GABA_BR$), two important members in the central nervous system.

Mechanisms of activation in GPCRs, how signals triggered by ligands bound at their extracellular domains are transmitted to their intracellular regions via 7TMD, are in general still poorly understood except for well-studied family A members. The activation models proposed for family A commonly suggest that helical movements within a receptor subunit are associated with its activation. In contrast, little is known for the activation mechanism of family C. This is partly because there are no receptors in this family whose full atomic coordinates are solved by X-ray crystallography. So far, this method has revealed the crystal structures of only the extracellular domains of mGluRs. No crystal structures reported for the $GABA_BR$ yet, making a proposal of its activation model difficult.

Fluorescence resonance energy transfer (FRET)-based technology emerged during the last decade has made the investigation of GPCR activation fruitful, largely owing to its ability to report events in time-lapse. To date, by introducing FRET-fluorophores at intracellular loop and C-terminus of receptor subunit, several studies have clarified the activation mechanism in family A receptors. All of them demonstrated FRET changes upon ligand-applications, validating the usefulness of this methodology. As for family C, the above method has also been applied for homodimeric mGluR1α by our group. In this study, they revealed the inter-subunit rearrangement, but not the intra-subunit structural change, takes its place when the receptor is activated by glutamate. Given the successful applications to GPCR research, he intended to use this approach to clarify the activation mechanism of heterodimeric $GABA_BR$, another member of family C GPCR. The present study examined $GABA_BR$ subunits GB1a & GB2 expressed in HEK293T cells at two different levels: inter-subunit FRET and intra-subunit FRET, all based on fluorescent proteins Cerulean and EYFP as a donor and an acceptor.

In the first part of experiments, the inter-subunit FRET studies, three different GB1a constructs bearing a Cerulean or EYFP at one of the first (i1), second (i2) or third (i3) intracellular loops were made and hereafter termed as GB1a-i1Cer/EYFP, GB1a-i2Cer/EYFP and GB1a-i3Cer/EYFP. In the same manner, GB2 constructs possessing an EYFP or Cerulean at one of the intracellular loops were made and named as GB2-i1EYFP/Cer, GB2-i2EYFP/Cer and GB2-i3EYFP/Cer. All combinations which lead to formation of heterodimer were coexpressed in HEK293T cells and changes in FRET were monitored during agonist-application. Among nine possible combinations, two pairs, GB1a-i2Cer & GB2-i2EYFP and GB1a-i2Cer & GB2-i1EYFP

demonstrated large FRET decreases of approximately 15 % upon 100 μM Baclofen application. The large FRET decrease from GB1a-i2Cer & GB2-i1EYFP and a subtle decrease from GB1a-i1Cer & GB2-i2EYFP indicate an asymmetric movement between the subunits.

The FRET decreases observed from the two pairs were further characterized and concluded to couple with agonist-binding based on the following findings: (1) the FRET decreases were reproducible after swapping the fluorophores, i.e., from the Cerulean & EYFP pairs to the EYFP & Cerulean pairs; (2) both FRET pairs exhibited counteraction, a synchronized positively-going Cerulean fluorescence trace and negatively-going FRET trace; (3) the EC_{50} values derived from concentration-response curves were 6.2 and 3.7 μM, similar to those in previous reports using the $GABA_BR$ expressed *in vitro*; (4) the FRET decreases were reversibly blocked by the antagonist CGP55845, indicating that the competitive antagonism at the receptor extracellular domain was monitored *in situ*; (5) when CGP7930, a positive allosteric modulator of the $GABA_BR$, was coapplied with the agonist, the FRET decrease was potentiated in the GB1a-i2 & GB2-i2, although this was not clear in the GB1a-i2 & GB2-i1.

In the second part of experiments, to seek intra-subunit conformational changes, GB2 and GB1a constructs all bearing EYFP at one of the intracellular loops and Cerulean attached at the C-terminus were made. First, a series of GB2 constructs commonly having Cerulean immediately after the coiled-coil domain were made (GB2-i1EYFP-T818Cer, GB2-i2EYFP-T818Cer and GB2-i3EYFP-T818Cer). However, when coexpressed with GB1a wild-type, these pairs did not respond to GABA or GABA with CGP7930. Next, a second series of GB2 constructs possessing Cerulean at more proximal position by complete deletion of the coiled-coil domains were made (GB2-i1EYFP-D769Cer, GB2-i2EYFP-D769Cer and GB2-i3EYFP-D769Cer). The new pairs, when coexpressed with GB1a S923 stop mutant, again failed to respond to ligands. GB2-i3EYFP-D769 exceptionally showed a subtle FRET decrease by agonist coapplied with CGP7930. Lastly, when the second series of GB2 constructs were coexpressed with GB1a I860 stop mutant, they again failed to respond to ligands except for GB2-i3EYFP-D769 which showed a subtle FRET decrease during the GABA application.

After confirming that none of the GB2 intra-subunit constructs displayed clear responses, intra-subunit FRET changes in GB1a were examined. New constructs, GB1a-i1EYFP-T872Cer, GB1a-i2EYFP-T872Cer and GB1a-i3EYFP-T872Cer, all have proximal Cerulean at the C-terminus by a complete deletion of the coiled-coil domain. However, when paired with GB2 wild-type, none of these constructs demonstrated a clear FRET changes upon ligand application. The situation was not changed when the same intra-subunit pairs were co-expressed with GB2 T749 stop mutant lacking the coiled-coil domain.

Only subtle or no changes were obtained in the intra-subunit FRET studies, which may had been caused by the insertion of fluorescent protein into the loops. Thus, positive control constructs were made. M_1 subunit of mAChR, a member of family A GPCR, was chosen as a backbone molecule. When an intra-subunit FRET pair was introduced into mAChR M_1 by an insertion of Cerulean at the third intracellular loop and attachment of EYFP at the C-terminus, the construct clearly showed agonist-induced FRET decrease upon an application of 10 μM

Oxotremorine M. This was reproducible when the positions of fluorophores were swapped. Hence, the reason why changes were not clearly observed from $GABA_BR$ intra-subunit FRET pairs cannot be ascribed to experimental limitations. Rather, the results suggest that both GB1a and GB2 subunits have more rigid structures compared with that of mAChR M_1.

The inter-subunit FRET study revealed an asymmetrical dissociation between GB1a and GB2 intracellular loops upon agonist-induced activation. In contrast, the intra-subunit FRET data suggest that the receptor activation does not involve major structural changes within each subunit. Combining the results obtained from two different levels, he concluded that the $GABA_BR$ activation by agonist is associated with an asymmetrical rearrangement of the two subunits GB1a & GB2 on the membrane. Moreover, the observed asymmetry highlights the qualitative difference between the mGluR1α and $GABA_BR$ activation mechanisms.

博士論文の審査結果の要旨

本論文は、G蛋白質共役型受容体(GPCR)の一種である代謝型GABA受容体($GABA_BR$)が、リガンド結合による活性化に際していかなる構造変化を起こすかという問いに、FRETを用いたアプローチにより答えようとしたものである。Family C GPCRに属する$GABA_BR$は、X線結晶構造が未だ解かれていないこともあって、その活性化メカニズムの全貌が明らかになっているとは言い難い。申請者は、この受容体を構成する2種のサブユニットであるGB1aおよびGB2の細胞内ドメインに蛍光蛋白を挿入することで、FRETペアを細胞膜上で形成させ、リガンド結合にともなう動的構造変化をFRET変化としてtime-lapseで捉えることを試みた。$GABA_BR$二量体の活性化にともなう構造変化は2つのレベル、すなわちサブユニット間のFRET (inter-subunit FRET)およびサブユニット分子内のFRET (intra-subunit FRET)に分けて調べられた。発現系としてはHEK293T細胞を用い、FRET imagingには全反射顕微鏡を使用した。

Inter-subunit FRETペアでは、GB1aの細胞内ループにCeruleanを挿入したものをドナーとし、同じくGB2の細胞内ループにEYFPを挿入したものをアクセプターとした。アゴニストとしてBaclofenを加えた際、GB1aの第2ループ(i2)およびGB2の第2ループ(i2)に蛍光蛋白が入ったペアと、GB1a-i2およびGB2-i1に蛍光蛋白が入ったペアで、10%以上のFRET減少が見られた。このような非対称な変化は、GB1a側をEYFPにしてGB2側をCeruleanとした組み合わせでも再現された。上記のGB1a-i2 & GB2-i2ペアとGB1a-i2 & GB2-i1ペアについて濃度作用曲線をとったところ、それらのEC_{50}は$GABA_BR$と共発現したチャネルの機能応答より得られたものとほぼ同じ範囲に収まるものであった。アゴニストにより惹き起こされた2種のFRET減少は、アンタゴニストCGP55845によりブロックされた。さらに、positive allosteric modulatorであるCGP7930を用いたところ、これはGB1a-i2 & GB2-i2由来のFRET減少だけをcoapplyにより増強することが判った。

Intra-subunit FRETペアとしては、GB1aおよびGB2のそれぞれのC末にCeruleanを、そして細胞内ループにEYFPを付けたものを作製した。Ceruleanの位置をよりEYFPに近づけてFRETを最適化するためにC末を短くした変異GB1aおよびGB2も用意して実験を行った。アゴニスト単独の投与およびCGP7930とのcoapplicationでは、一連のGB1a intra-subunitペアからは明瞭なFRET変化は生じなかった。GB2 intra-subunitペアのシリーズでもほとんど同様の傾向であった。これに対し、コントロールとして作製したムスカリン性アセチルコリン受容体のM_1サブタイプ(mAChR M_1)を土台としたintra-subunitペアでは、アゴニスト投与にともなって明確なFRET減少が見られた。これらの知見から、mAChR M_1と比較するとGB1aおよびGB2は相対的に剛直な分子構造を持っているものと考えられる。

以上、inter-subunitおよびintra-subunit FRETの結果から、$GABA_BR$の活性化時にはサブユニット同士が非対称に離れるという再配置が起き、その一方で、サブユニット分子内における構造変化はわずかなものであろうと結論付けた。既に同様なアプローチによる研究が同じくFamily Cに属する代謝型グルタミン酸受容体についてなされているが、この受容体ではお互いに離れる細胞内ループと近づく細胞内ループが検出され、2つのサブユニットの間では対称的な効果が認められている。本論文で新たに示された$GABA_BR$のサブユ

ニット再配置の様式は、これとは質的に大きく異なるものであり、大変興味深い。多くのFRET ペアを用いて網羅的な実験が行われており、結果の信頼性も高い。従って、本論文は学位論文として十分な水準にあることが審査員全員の一致した意見である。