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学 位 論 文 名	アゴニスト依存的または非依存的な二量体受容体と β-アレスチン 2 の相互作用の解析
論 文 審 査 委 員	(委員長) (委 員)

論文内容の要旨

1 研究目的

G-protein couple receptors (GPCRs) function as a monomer, as well as a dimer. However, information is limited on interaction among three molecules, which are composed of dimerized receptor and β -arrestin 2. Therefore, we decided to develop a method to detect their interactions. Vasopressin V1a receptor (V1aR) and V1b receptor (V1bR) belong to the member of GPCRs. They were used as a model because of similar structure but different characters. Previous studies have demonstrated that arginine vasopressin (AVP) is involved in morphine-induced anti-nociception through V1bR and μ -type opioid receptors (MOR), which are co-localized in the rostral ventromedial medulla (RVM). The interaction between dimerized V1bR-MOR and β -arrestin 2 has been suggested but not examined. Hence, we studied the interaction between homo- or heterodimer receptors and β -arrestin 2.

2 研究方法

We considered that NanoBiT and NanoBRET methods can be used to investigate the three-molecule interaction among dimer receptor and β -arrestin 2. NanoLuc[®] Binary Technology (NanoBiT) is a method for detection of protein-protein interaction. Two parts of NanoBiT, a Large BiT (LgBit) and a Small Bit (SmBit), complementarily form a whole Nanoluciferase. When LgBit or SmBit were connected with genes of interest and expressed in cells, luminescent light indicates interaction of the two proteins. Therefore, carboxyl- (C-) termini of the mouse V1aR, V1bR and MOR were connected to LgBit or SmBit. The β -arrestin 2 was connected with Venus at amino- (N-) terminus. NanoBRET method uses the bioluminescent resonance energy transfer between a donor of Nanoluciferase protein and an acceptor of fluorescent protein for detection of protein-protein interaction. If receptor dimer interacted with β -arrestin, NanoBRET will occur between NanoBiT Nanoluciferase and Venus. We measured NanoBRET in cells stimulated with or without agonists of various concentrations. NanoBRET value was analyzed by light intensities at 530/480 nm. HiBit

couples with LgBit with higher affinity than SmBit. Using the HiBit, the effect of interaction with high affinity was examined.

3 研究成果

Intracellular calcium responses of V1aR-SmBit and V1aR-LgBit were comparable to those of V1aR. In addition, the intracellular calcium responses of V1bR-LgBit and V1bR-SmBit were similar to those of V1bR. Therefore, LgBit and SmBit, fused to the V1aR or V1bR, did not impede the receptor function.

Strong luminescence signals with gradual increase were detected in cells co-expressing receptor-LgBit and receptor-SmBit. Our results clearly indicated that the homodimerization of receptors occurred without agonist in our system. Preparation of membrane fraction from the cells expressing V1aR-LgBit and V1aR-SmBit, and addition of the substrate directly to membrane samples generated luminescence signal with gradual increase. Gradual increase in luminescence was seen also in constitutively associated cAMP-dependent protein kinase type II- α regulatory subunit and catalytic subunit pairs. These results suggested that substrate might increase activity of Nanoluciferase, which was formed by LgBit and SmBit.

The MOR, V1aR and V1bR tagged with whole luciferase (Nluc) or split Nanoluciferase (LgBit and SmBit) were expressed in HEK cells. The agonists, DAMGO and AVP, were used for stimulation of MOR and vasopressin receptors, respectively. Our results showed that intensities of luciferase signals were not changed by agonist stimulations.

We co-expressed V1aR and P2X2aR in HEK cells to examine whether non-specific binding could occur between V1aR and P2X2aR. The results showed that the luciferase signals from cells expressing V1aR and P2X2aR were in the background level, compared to the high signals from P2X2aR homomers and V1aR homomer.

Interaction between V1aR-Nluc and β -arrestin 2-Venus was initiated by agonist and increased in an agonist-dependent manner. In contrast, basal BRET signal without agonist was already high in cells expressing both V1b-Nluc and β -arrestin 2-Venus, indicated that the interaction occurred without agonist stimulation. Furthermore, agonist-dependent increase in BRET was not detected between V1b-Nluc and β -arrestin 2-Venus. Therefore, we explored an experimental condition, in which two parts of a split Nanoluciferase (LgBit and SmBit) were connected to the C-termini of V1b or V1a and interaction was examined between receptor homodimer and β -arrestin 2 in NanoBit-NanoBRET system. Our data indicated that the split Nanoluciferase method increased the sensitivity to detect agonist-dependent interaction between dimerized V1aR or V1bR and β -arrestin 2.

Reduction of the plasmid amounts for transfection of receptor-LgBit and receptor-SmBit with keeping those of β -arrestin 2-Venus at constant level improved apparent BRET response, which were generated from receptor dimer and β -arrestin. In these experiments, although plasmid for β -arrestin-Venus was used with fixed amount for transfection, fluorescence from β -arrestin-Venus actually decreased with increase in the luminescence of receptor dimers. At

this point we speculated that relative expression levels of receptor-connected luciferase and β -arrestin-Venus might be an important factor for sensitive BRET detection.

We next changed the amount of transfected plasmid for β -arrestin 2-Venus with fixed level of the receptor-LgBit and -SmBit. The results showed that increasing the amount of plasmid for β -arrestin 2 increased the BRET signal in an agonist-dependent manner. Basal BRET levels without AVP administration were higher in V1bR homodimers than those in V1aR, indicating that V1bR homodimers were more likely to bind with β -arrestin 2 at basal condition. Although fixed amounts of plasmids for receptor-LgBit and receptor-SmBit were used, luminescence intensities were changed in accordance with changing expression levels of β -arrestin 2-Venus: luminescence from receptor dimers were tended to be low, if expression of β -arrestin 2-Venus was high and resulted in high amplitude of the agonist-induced BRET signal.

When expressed with V1bR-LgBit, V1bR-HiBit generated stronger luminescence than V1bR-SmBit. In contrast, maximum BRET values of cells expressing V1bR-HiBit were lower than those expressing V1bR-SmBit, although reducing levels of V1bR-LgBit and V1bR-HiBit also increased maximum levels of BRET signal.

In V1aR-Nluc expressing cells, basal and agonist-induced BRET increased when β -arrestin 2-Venus was expressed together with V1aR-Nluc. In contrast, the BRET signals at 530/480 nm did not change in a wide range of expression levels of V1aR-Nluc, if plasmid for β -arrestin 2-Venus was not included in transient expression.

We compared receptor dimers composed of V1bR-LgBit-MOR-SmBit and those of V1bR-SmBit-MOR-LgBit in terms of efficiency to couple with β -arrestin 2. In both agonists for V1b and MOR, V1bR-LgBit-MOR-SmBit heteromer generated larger responses than opposite combination of receptor tags.

4 考察

Our experiment demonstrated that the receptors constitutively dimerized independent of agonist. Interaction between receptor C-termini was stable in the receptor homomers and V1b-MOR receptor heteromers. Therefore, agonist-induced changes in BRET levels in our experiments indicated interactions between receptor and β -arrestin 2.

When expressed with seven transmembrane receptors, such as V1a or MOR, the P2X2aR connected with split Nanoluciferases produced only background signal. Therefore, our experimental condition detected receptor-receptor interaction of the same structural group.

For the basal BRET levels, V1b homomeric receptors interacted with β -arrestin 2. In contrast, we found that interaction between V1a homomer and β -arrestin 2 at basal was dependent on the ratio between V1aR and β -arrestin 2. AVP stimulation further increased the BRET values in V1a and V1b homomer receptors. We also applied this sensitive method to the analysis of interaction between MOR-V1bR heteromer and β -arrestin. V1bR-LgBit plus MOR-SmBit had larger BRET signals than those of opposite receptor tags.

Both reducing the receptor plasmid and increasing the β -arrestin 2 plasmid for transfection improved BRET responses. Brightness of Nanoluciferase made this condition be possible to detect even in the low expression level. High β -arrestin 2 level relative to V1bR in our experiment might be artificial condition compare to native tissue. However, our sensitive method has a clear advantage to search for partial agonists and antagonists. Furthermore, the V1bR-HiBit developed lower BRET signal than V1bR-SmBit did. This result indicated that increasing interaction between receptor C-termini did not improve BRET between V1bR homomer and β -arrestin 2.

5 結論

Dimerization of receptors should be carefully examined prior to the examination of BRET. Thereafter, agonist-dependent interactions between dimerized receptor and β -arrestin were validated in our study. From the results on the swapping a pair of the split Nanoluciferase, both combinations of tagging to the receptor heteromers should be examined on its efficacy. Agonist effect on MOR-V1b receptor heteromer will allow us to search for an antagonist, which can reduce BRET response from the dimerized receptors and β -arrestin 2 and reduce tolerance to morphine analgesia.

論文審査の結果の要旨

本研究は GPCR のホモダイマーあるいはヘテロダイマー形成と arrestin カップリングにおけるリガンドの役割を vasopressin receptor を用いて検討したものである。具体的には、HEK 細胞への強制発現系を用い、Vasopressin V1a 受容体、vasopressinV1b 受容体のホモダイマー形成と arrestin とのカップリング、vasopressin V1b 受容体とオピオイド受容体のヘテロダイマー形成と arrestin とのカップリング、そして、ダイマー形成と arrestin カップリングにおけるリガンド依存性を検討している。この検討に、候補者は、2 つのタンパク質の相互作用を測定するためのルシフェラーゼと蛍光単白質による生物発光共鳴エネルギー移動法である NanoBRET® Assay 法、分離型のルシフェラーゼを用いた NanoBiT 法とを組み合わせ、3 つの分子の相互作用を検討している。

NanoBiT 法では、分離型ルシフェラーゼをそれぞれ異なる分子に付加させ、結合によりルシフェラーゼ酵素活性が出現することで、2 つの異なる分子の結合を検出する。そこでこのタグ付加により受容体の機能が阻害されないことをリガンド投与による細胞内カルシウム濃度上昇で確かめている。さらに NanoBit 法で V1a 受容体、V1b 受容体、mu-opioid 受容体が多量体を形成すること確認し、P2X2a と V1a 受容体間では多量体形成は生じないことを確かめた。このルシフェラーゼ活性を指標とした多量体形成にはアゴニストの投与は影響を及ぼさないことを見ている。次に、受容体と arrestin との結合におけるアゴニストの効果を BRET 法で検討している。分離型ルシフェラーゼ法を用いると V1a 受容体、V1b 受容体ともに、アゴニスト依存性の多重受容体と arrestin のカップリングの増加が観察された。このアゴニスト依存性の増加は用いる受容体のプラスミド量を減少させるほど、また arrestin のプラスミド量を増やすと効率よく観察することが

できることを見出した。

最後に分離型ルシフェラーゼを mu-opioid 受容体と V1b 受容体に付加し arrestin とのカップリングにおけるアゴニストによる増強作用も観察している。またこの時、これまでの nano ルシフェラーゼを用いた方法では検出できなかったアゴニスト依存性カップリング増加が分離型ルシフェラーゼで検出できること、分離型ルシフェラーゼの組み合わせによりアゴニスト依存的増加の効率が変わることも示した。

本研究は3つの蛋白質の相互作用を検討する方法として、NanoBRET® Assay 法と、分離型のルシフェラーゼを用いた NanoBiT 法を組み合わせたもので、至適発現量を含む方法論の検討も含めて行っており、新規性がある。

研究全体の位置づけを示す文章の追加、一部文言の変更を示唆され適切に訂正された。訂正された学位論文は、本学学位論文としてふさわしいと判断された。

最終試験の結果の要旨

申請者は、GPCR の働き、蛋白質相互作用を検討する方法についての NanoBRET®法、分離型のルシフェラーゼを用いた NanoBiT 法とこれらを組み合わせた方法についての概説した後、バゾプレシン V1a 受容体、V2 受容体、mu-opioid 受容体の多量体形成と arrestin カップリング、これらにおけるアゴニストの効果についてデータを説明した。

研究全体の意義、V1a 受容体、V1b 受容体と mu-opioid 受容体を用いた実験を行う理由、用いた方法の有用性とその限界、G 蛋白質を強制発現させていないことの影響、細胞内領域の C 末にタグ付加したことの実験系における影響、今回の in vitro データから生理的働きについて推察できること、について質疑があり、申請者は真摯な態度で適切に応答した。申請者は研究関連領域の背景についての知識も十分もっており、本学学位授与にふさわしいと判断された。