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学位(専攻分野)	博士(理学)
学 位 記 番 号	総研大甲第 2121 号
学位授与の日付	2019 年 9 月 27 日
学位授与の要件	生命科学研究科　生理科学専攻 学位規則第6条第1項該当
学 位 論 文 題 目	ATP- and voltage-dependent gating of P2X2 receptor analyzed by voltage-clamp fluorometry using fluorescent unnatural amino acid
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(Form 3)

Summary of Doctoral Thesis

Name in full Andriani, Rizki Tsari

Title ATP- and voltage-dependent gating of P2X2 receptor analyzed by voltage-clamp fluorometry using fluorescent unnatural amino acid

P2X2 is a homotrimeric ligand-gated ion channel activated by extracellular ATP. P2X2 receptor is widely distributed in variety of cell types with main distribution in smooth muscles, central nervous system (CNS), retina, chromaffin cells, autonomic and sensory ganglia. P2X2 receptor regulates neurotransmission by both pre- and post-synaptic actions. Based on the crystal structure of zebrafish P2X4 and human P2X3, P2X purinoreceptors are known to have a topology with two transmembrane (TM) domains, a large extracellular ligand binding loop, and intracellular N and C termini. The extracellular domain connecting the two TMs constitutes the largest part of the polypeptide.

One of the interesting characteristics of P2X2 receptor is that it has a complex gating consists of (1) [ATP]-dependent gating and also (2) voltage-dependent gating in spite of the absence of a canonical voltage sensor domain, in clear contrast to the typical voltage-gated channels which have a voltage sensor domain within the structure. It remains unknown how the structural rearrangements occur during the voltage dependent gating. Besides, the detail of the structural rearrangements upon ATP binding in the pore region remains controversial. It is because there is a discrepancy between the two ATP-bound open state from the two solved crystal structures, *zf*P2X4 and *h*P2X3. The *h*P2X3 structure showed longer transmembrane domains than *zf*P2X4 and it includes cytoplasmic domains. The present study aims at analyzing the structural rearrangements of the rat P2X2 receptor upon (1) ATP- and (2) voltage-dependent

gating, by voltage-clamp fluorometry (VCF) using fluorescent unnatural amino acid (fUAA) probe.

A usage of fUAA as a probe, made it possible to label any residues within the protein including intracellular region which is not accessible by conventional VCF fluorophores such as Alexa-488 maleimide. Moreover, direct incorporation of the fUAA will increase the labelling efficiency and also prevent non-specific labelling. The fUAA used here, named *3-(6-acetylnaphthalen-2-ylamino)-2-aminopropionic acid* (Anap), was incorporated into the rat P2X2 protein by using *in vivo* non-sense suppression method where the tRNA Anap-CUA and tRNA-synthetase pair is used to introduce Anap in amber nonsense codon mutation. TAG mutation was introduced to various positions in P2X2 receptor, one at a time including extracellular domain, extracellular linker, transmembrane domains, as well as intracellular domains. A plasmid DNA containing tRNA Anap-CUA and tRNA-synthetase pair was injected to the nucleus of *Xenopus laevis* oocyte. Subsequently, the rat P2X2 cRNA in which the target site was mutated to a TAG codon and Anap were co-injected to the cytoplasmic region of the oocyte on the following day. In addition to that, to improve the VCF recording optical signal by decreasing the intrinsic background fluorescence of oocytes, a small molecule kinase inhibitor named HG-9-91-01 (SIK inhibitor) was applied. ATP- and voltage-evoked current as well as Anap fluorescence signal in the functional Anap mutant P2X2 receptors were successfully recorded simultaneously.

VCF analyses using Anap as a probe to overcome the limitations by the usage of conventional fluorophore with the application of SIK inhibitor to improve the VCF optical signal brought the following findings. (1) Anap was successfully incorporated into P2X2 receptor shown by simultaneously recorded (i) ATP- and voltage- evoked current as well as (ii) Anap fluorescence signal. (2) SIK inhibitor treatment improved VCF-fUAA optical signal. (3) Voltage-dependent fluorescence changes of Anap was observed only at Ala337 and Ile341 in TM2 domain. (4) The changes showed a linear

voltage-dependence, and exhibited fast kinetics in ms order which might indicate a phenomenon related to electrochromic effect. (5) The observed electrochromic effect at Ala337 and Ile341 implied that there is a focused electric field at these positions. (6) Voltage-dependent fluorescence change at Ala337 was larger in the absence of ATP than in the presence of ATP, reflecting the ATP-dependent change of the focused electric field. (7) Mutagenesis studies at Ala337 and Phe44 suggested that the interaction between Ala337 in TM2 and Phe44 in TM1 in the open (ATP-bound) state is important for the complex gating of P2X2 receptor.

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Results of the doctoral thesis screening

博士論文審査結果

Name in Full
氏　名　Andriani, Rizki Tsari

Title
論文題目　ATP- and voltage-dependent gating of P2X2 receptor analyzed by voltage-clamp fluorometry using fluorescent unnatural amino acid

プリン作動性 P2X2 受容体は細胞外 ATP をリガンドとするホモ３量体のリガンド作動性カチオンチャネルであり、中枢神経系のシナプス神経伝達をはじめ、様々な細胞種の機能制御に関わることが知られている。P2X2 受容体はいわゆる電位依存性チャネルに共通して保存されている膜電位センサードメイン配列を持たないにも関わらず、ATP 存在下で電位依存的に活性化されるという特徴を有する。しかし、その膜電位依存的活性化の分子基盤は未解明であった。本論文は、蛍光性の非天然アミノ酸（3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid (Anap)）を用いた voltage-clamp fluorometry 法とチャネル電流の同時計測により、P2X2 受容体のリガンド依存的かつ電位依存的な活性化（complex gating）の分子機構を解明した研究である。

出願者は、tRNA-Anap-CUA と Anap tRNA synthetase をコードするプラスミドを導入したアフリカツメガエル卵母細胞に、Anap を挿入したいアミノ酸残基部位の塩基配列を TAG に変異させたラット P2X2 mRNA と Anap をインジェクションする *in vivo* ナンセンス抑制法を用いて、特定の位置に Anap を取り込んだ P2X2 タンパク質を細胞膜に発現させた。さらに、卵母細胞のバックグラウンド蛍光を減弱させる小分子化合物（SIK 阻害剤）を適用することで、P2X2 の膜電流変化と蛍光変化を同時計測する最適条件を確立した。膜電位感知に関係する可能性が想定された 96 カ所のアミノ酸残基にひとつずつ Anap を導入した 96 種類の P2X2 点変異体を用いた解析により、第二膜貫通領域の Ala337Anap と Ile341Anap においてのみ電位依存性の蛍光変化が観察された。その蛍光強度は膜電位変化に伴いリニアに変化し、またミリ秒単位の速いキネティクスを示した。この特徴は、蛍光強度の変化が、タンパク質の構造変化に伴うものというより、膜電位を直接反映するエレクトロクロミック効果によるものであること、すなわちこの位置に電場が強く集約していることを示唆する。Ala337 における電位依存的な蛍光変化は ATP 存在下よりも ATP 非存在下で大きかったことから、Ala337 と Ile341 の位置の電場が ATP 依存的に変化することも示された。さらに、第一膜貫通領域にある Phe44 の点変異体解析の結果から、ATP 結合（開口）状態における第一膜貫通領域の Phe44 と第二膜貫通領域の Ala337 との相互作用が P2X2 受容体の complex gating に重要であることを明らかにした。

以上の結果は、既存の膜電位センシング機構とは異なる P2X2 受容体内の集約した電場に依存した complex gating の分子機構を示唆した画期的な知見であり、その生理学的意義は極めて大きいことから、全会一致で合格と判定した。