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学位論文題目	Identification of critical amino acid residues of the $P2X_2$ receptor channel towards the mechanism of the voltage-and [ATP] -dependent “gating”
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論文内容の要旨

$P2X_2$ is a non-selective cation channel, activated by extracellular ATP. It is one of the members of P2X receptor family which are widely distributed in various tissues and play important roles in many physiological functions. Although it is a ligand gated channel, its structure differs from other cys-loop and glutamate receptor channels. P2X receptor is trimeric with a large extracellular loop of approximately 280 amino acid residues and has its N and C termini intracellular. It has two transmembrane (TM) regions with no canonical voltage sensor.

In spite of the absence of a voltage sensor domain, $P2X_2$ receptor channel has an unexpected voltage dependent gating, showing a gradual increase in the inward current upon hyperpolarization in the steady state after ATP application. When the activation phase of gating and the tail current were analyzed, an apparent shift of conductance-voltage (G-V) relationship to depolarized potentials and an acceleration of the activation phase with the increase in the concentration of ATP ([ATP]) were observed. The results revealed that gating of $P2X_2$ receptor channel depends on both voltage and [ATP]. Furthermore, voltage and [ATP] dependent gating of $P2X_2$ receptor channel could be successfully reproduced by a three-state model which consists of a fast ATP binding step and a rate limiting voltage dependent gating step, using experimental data.

In the next step, a mutagenesis approach was used to identify the amino acid residues which are responsible for the voltage and [ATP] dependent gating. Since there were an apparent [ATP]-dependent shift of $V_{1/2}$ values of G-V relationship to depolarized potentials and a voltage induced acceleration of activation phase, he hypothesized that the positively- charged ATP binding pocket, together with ATP, might act as a voltage sensing particle contributing to the voltage dependent step. With this hypothesis, K308A/R, K69A/R, K71A/R, R290A/R mutations were introduced at the previously reported ATP binding site. K308R mutant showed G-V relationship accumulated at hyperpolarized potentials without any clear [ATP] dependent shift to depolarized potentials. Furthermore, the activation speed was faster than WT without an apparent acceleration by [ATP]. Similar phenotypes were also observed in other ATP binding site mutants. The results could be summarized that the equilibrium is inclined to the closed state with faster kinetics to reach equilibrium suggesting an increase in the off gating rate in the voltage dependent step. Therefore, by using the same three-state model, a simulation study was performed with a high off gating rate. Simulation results were similar to the experimental results from the ATP binding region mutants. These results suggest that residues of the ATP binding site

contribute to voltage-dependent conformational changes of $P2X_2$ receptor molecule.

A similar analysis of voltage dependent gating was performed in WT with adenosine diphosphate (ADP) and diadenosine tetraphosphate (AP_4A), two different agonists having different structure and electrostatic charge. Four phosphate groups of AP_4A, having 4 free negative charges are connected to two adenine rings on each end. ADP with two phosphate groups has 3 negative charges, one less than ATP. Both agonists had relatively lower potency than ATP. Voltage dependent activation phase and tail current analyses with ADP showed similar results with the ATP responses. The Hill coefficient from the dose response curve of ADP was also close to 2, similar to ATP response. The results from the ADP responses suggest that the total free charge of the ligand solely, does not determine the voltage dependent gating behaviour. On the other hand, when AP_4A, a relatively larger and less flexible molecule was used, G-V curves accumulated at hyperpolarized potentials without any apparent shift to depolarized potentials. The activation speed was relatively fast and did not show any apparent acceleration by further increase in [AP_4A]. Voltage dependent gating property in response to AP_4A resembled to the gating observed in ATP binding region mutants. Moreover, Hill coefficient from the dose response curve of AP_4A was close to 1 suggesting a clearly different ligand-receptor interaction from those of ADP and ATP. The Z values of the G-V curves from AP_4A, ADP and ATP responses were similar. The results suggest that rather than the total free charge of the ATP binding pocket per se, the molecular and conformational structure of the ligand-ligand binding site complex contributes to the voltage dependent gating behaviour.

Furthermore, amino acid residues F44, Y43 and Y47 close to the extracellular region of TM1, and I328 and T339 in TM2 were identified to be critical for the voltage dependent gating. Mutations of these amino acid residues (F44C/A Y43A, Y47C in TM1 and I328C/S/L and T339S in TM2) made the channel activate slowly upon hyperpolarization at low [ATP]. At high [ATP] the channel became constitutively active and voltage dependent gating was abolished. The results could be summarized that the equilibrium is inclined to the open step with slower kinetics to reach equilibrium suggesting a decrease in the off gating rate in the voltage dependent step. By using the same model, experimental results for the TM region mutants were reproduced by decreasing the off gating rate.

Finally, analyses of double mutants were performed which have an ATP binding region mutation with a high off gating rate and a TM1 or TM2 mutation with a low off gating rate (eg; K308R/T339S and K308R/F44C). The results revealed a phenotype similar to WT, suggesting a mutual interaction of these residues in the same rate limiting voltage dependent gating step.

Taken together, the present work demonstrates that the amino acid residues at the putative ATP binding site and at the extracellular side of two TM regions are critically involved in the voltage dependent gating, and that the molecular interaction of ATP with the ligand binding environment is transmitted as a signal for the voltage dependent gating to the extracellular side of TM1 and TM2 where critical conformational changes occur.

博士論文の審査結果の要旨

ATP 受容体チャネル $P2X_2$ は、細胞外 ATP によって活性化される非選択性カチオンチャネルであり、2 回膜貫通型のサブユニットの 3 量体として構成される。これまでに Keceli 氏らは、ツメガエル卵母細胞を発現系として用い、ATP 投与後の定常状態における $P2X_2$ チャネル電流と膜電位との関係を定量的に解析し、(1) 膜電位依存性のゲート機構が存在し過分極電位で開きやすいこと、(2) ATP 濃度に依存してコンダクタンス-膜電位 (G-V) 関係が脱分極側にシフトし、また活性化相が加速することを見いだした。さらに、(3) 速い ATP 結合ステップと、律速段階となるゲートステップで構成される 3-state モデルを仮定し、種々の電位、ATP 濃度でのゲートステップの速度定数 k_{on} と k_{off} を決定した。さらに、これらの値と、既報告の膜電位に依存しない ATP 結合の速度定数, k_{bind} と k_{unbind} を用いてシミュレーションを行い、膜電位依存性ゲート機構が ATP 濃度にも依存するという実験結果を再現することに成功した (Fujiwara, Keceli, Nakajo, Kubo (2009) Journal of General Physiology)。

本学位論文において、申請者 Keceli 氏は、多くの電位依存性チャネルに共通する電位センサーを欠く ATP 受容体チャネルが膜電位依存性を有する分子基盤を明らかにするために、変異体解析等を行った。(1) ATP 結合部位と同定されている領域の変異体 (K308A/R, K69A/R, K71A/R, R290A/R) のうち、K308R では電位依存性が過分極側に大きくシフトし活性化速度が速かった。この性質は、モデルでゲートステップの k_{off} を増加させることにより再現できた。(2) 次に、ATP による活性化に関与する膜貫通部位の細胞外側端に位置するアミノ酸残基の変異体 (F44C/A, Y43A, Y47C, I328C/S/L, T339S) の解析を行った。T339S 等は、低い ATP 濃度では遅い膜電位依存的活性化を示し、高い ATP 濃度では膜電位に依存しない恒常的活性化を示した。この性質は、モデルにおいてゲートステップの k_{off} を減少させることにより再現できた。(3) k_{off} に逆向きの変化を与えた K308R と T339S の 2 重変異体は、野生型 $P2X_2$ に極めて近い性質を示した。このことから、膜電位依存的ステップにおいて、ATP 結合部位と膜貫通部位の二つの領域が相互作用していることが示唆された。(4) さらに、ATP と電荷、大きさが異なるアゴニスト、ADP, AP_4A の野生型 $P2X_2$ に対する作用を解析したところ、ADP の効果は ATP と類似し、AP_4A の効果は、K308R に対する ATP の効果と類似していた。このことは、ATP の結合部位の電荷を保存した変異 K308R で際だった変化が観察されることと併せて、ATP-ATP 結合部位複合体の電荷だけでは決定されておらず、結合の様式等が、膜電位依存的ゲート機構に影響していることを示唆した。

以上の実験結果から、ATP 受容体チャネル $P2X_2$ の膜電位依存的ゲートには、ATP-ATP 結合部位複合体と、膜貫通部位細胞外側端が複合的に寄与していることが示された。ATP-ATP 結合複合体が転位し、膜貫通部位細胞外側端に作用し、ゲート開口につながる構造変化を

トリガーすること、その作用部位が膜電場内に位置するため膜電位依存性を有することが示唆された。

本論文は、変異ATP受容体の詳細な機能解析とシミュレーションから、特に電位に依存するゲートステップの分子機構に新たな知見を加えるものであり、博士論文に値するものと審査員一致で判断された。