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研究課題名（和文）The physiological ligand and activation mechanisms of an orphan metabotropic receptor Prrt3

研究課題名（英文）The physiological ligand and activation mechanisms of an orphan metabotropic receptor Prrt3

研究代表者

陳　以珊（Chen, I-Shan）

生理学研究所・分子細胞生理研究領域・特任助教

研究者番号：４０７５７７７０

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研究成果の概要（和文）：Prrt3は、オーファン代謝型受容体である。Prrt3に関する先行論文は無いため、Prrt3の生理的役割や分子機能は全く未知である。本研究課題では、Prrt3のリガンドの同定と活性化機構の解明を目指して実験を行った。その結果、（1）Prrt3はGi/o蛋白質と結合し、Gq蛋白質と結合しないこと、（2）Prrt3のN末端細胞外領域の切断は分子を活性化しないこと、（3）ムスカリン性アセチルコリン受容体アゴニストに属する数個の化合物がPrrt3を活性化するが、アセチルコリンは活性化しないこと、（4）抗寄生虫剤であるIvermectinはGIRKチャネルを直接活性化することが明らかになった。

研究成果の概要（英文）：Prrt3 is an orphan family C metabotropic receptor. There is no publication concerning Prrt3 and the physiological roles and functioning mechanisms of Prrt3 are totally unknown. In the present study, we aim to clarify the physiological ligand and activation mechanisms of Prrt3. We performed molecular biological, electrophysiological and [Ca2+]i imaging experiments using Xenopus oocytes and HEK293 cells, and we observed the following results: (1) Gi/o, but not Gq, is coupled with Prrt3; (2) Prrt3 is not activated by the cleavage of its N-terminal extracellular domain; (3) some muscarinic receptor agonists, but not ACh, slightly activates the Prrt3; (4) we happened to identify a novel activator of GIRK channel, ivermectin.

研究分野：　生理学

キーワード：　Ligand　GPCR

１．研究開始当初の背景

Proline rich transmembrane protein 3 (Prrt3) is an orphan G-protein-coupled receptor (GPCR) which contains a large N-terminal extracellular domain and seven transmembrane domains (Fig. 1). Although Prrt3 possesses a large extracellular domain like other family C GPCRs do, its physiological roles and functioning mechanisms are totally unknown.

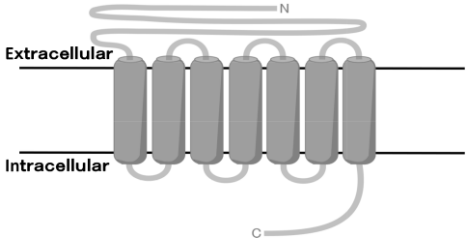

Figure 1. Membrane topology of Prrt3. Prrt3 contains seven transmembrane domains and a large N-terminal extracellular domain.

We have found followings so far:
(1) By immunohistochemical experiments, we observed that Prrt3 is highly expressed in brain including thalamus, hippocampus, cortex and substantia nigra in mouse (Fig. 2).

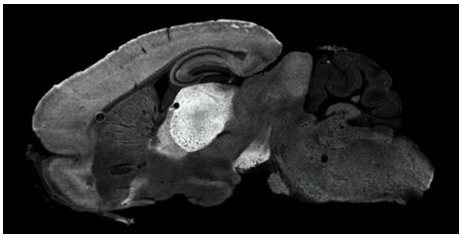

Figure 2. Prrt3 expression in WT mouse brain fresh frozen section. (by Dr. Konno, Hokkaido University).

(2) We also observed that Prrt3 is most likely expressed in the presynaptic terminal and/or periphery of post synapse but not in the postsynaptic density.
(3) Behavioural experiments using herterozygous Prrt3 knock out (KO) mice indicated that Prrt3 is involved in the retention of spatial memory and fear conditioning memory.
(4) Homozygous Prrt3 KO mice showed high mortality.
(5) Western blot (WB) experiments using wild-type (WT) mice suggested that the N-terminal extracellular domain of Prrt3 is partly cleaved from its transmembrane domain.
(6) We examined the effect of serine proteases, which are known to cleave peptide bonds at lysine or arginine residues. Results of immuno precipitation and WB experiments using C-terminal Prrt3 antibody suggested that co-expression of Prrt3 with a proprotein convertase furin only partly generates the cleaved form of Prrt3 in HEK293 cells.
(7) Mutations of arginine at position 343 and 345 (conserved among various species) into serine partly inhibited the generation of the cleaved form of Prrt3.
(8) The $G_{i/o}$ class of G_{α} is identified as a co-immunoprecipitating protein with Prrt3 by mass spectrometry (MS) analyses.
(9) We recently examined the effect of various neurotransmitters and their related chemicals, and observed that a muscarinic ACh receptor agonist, oxotremorine-M (Oxo-M), but not ACh, slightly activates the $G_{i/o}$ protein-gated inwardly rectifying K^+ (GIRK) currents in Prrt3-expressing *Xenopus* oocytes (Fig. 3).

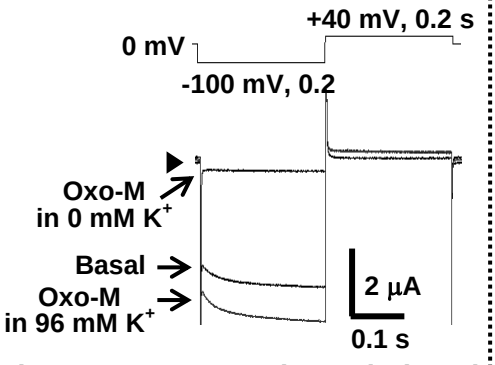

Figure 3. Oxotremorine-M-induced GIRK currents in Prrt3-expressing oocytes.

２．研究の目的

Since there is no publication concerning Prrt3 and its physiological ligand remains unknown, we aim to clarify the physiological ligand and activation mechanisms of Prrt3.

３．研究の方法

In order to clarify the physiological ligand and activation mechanisms of Prrt3, we performed experiments as follows:
(1) To identify the class of Prrt3-coupled G-proteins by electrophysiological experiments and $[Ca^{2+}]_i$ imaging.
(2) To identify the activated form of Prrt3

by producing the cleaved form of Prrt3 using proteases or mutagenesis technique.
(3) To identify the physiological activator of Prrt3 by ligand library screening.
See the results for more detail.

４．研究成果

(1) Identification of the class of Prrt3-coupled G-proteins

According to our previous MS analyses and electrophysiological experiments, $G_{i/o}$ class of G_{α} couples with Prrt3. As Oxo-M only slightly activates GIRK currents, we speculate that Prrt3 may partly couple with $G_{i/o}$ and partly couple with other class of G_{α}. To prove this hypothesis, we examined the G_q-mediated signaling by recording the effect of Oxo-M on Ca^{2+}-activated Cl^- currents (CaCC) in oocytes using two-electrode voltage clamp. We observed that Oxo-M induces CaCC currents in G_q-coupled M1 muscarinic receptor (M1R)-expressing oocytes, but it fails to induce CaCC currents in Prrt3-expressing oocytes, suggesting that G_q does not couple with Prrt3 (Fig. 4).

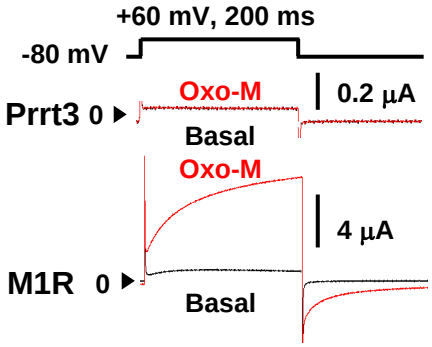

Figure 4. Oxotremorine-M-induced CaCC currents in Prrt3- or M1R-expressing oocytes.

To further confirm the coupling between Prrt3 and $G_{i/o}$, we transfected Prrt3 with G_{α}16z44, which links $G_{i/o}$-receptors activation with the G_q-mediated signaling into HEK293 cells and measured the G_q-mediated $[Ca^{2+}]_i$ increase by $[Ca^{2+}]_i$ imaging using fura-2 AM. We observed that application of Oxo-M induced fluorescent Ca^{2+} signal increase, suggesting that $G_{i/o}$ is coupled with Prrt3 (Fig. 5).

(2) Identification of the activated form of Prrt3: the full-length form vs. the cleaved form

Our WB analysis suggests that the

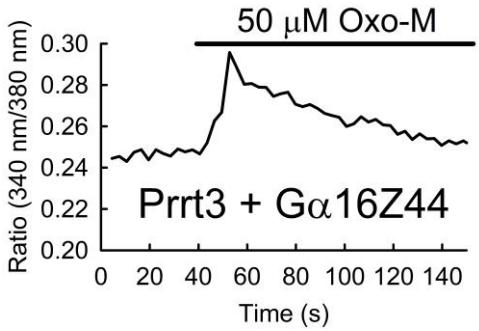

Figure 5. Effects of Oxo-M on fluorescent Ca^{2+} signal in HEK293 cells expressing Prrt3 and G_{α}16z44.

N-terminal extracellular domain of Prrt3 is cleaved from its transmembrane domain in the brain. However, there is no information indicating which conformation (the full-length form or the cleaved form) is the activated form of Prrt3. Here we produced the cleaved form of Prrt3 by two methods: (1) co-expression of Prrt3 with furin in oocytes; (2) truncation of the N-terminal extracellular domain by mutagenesis (Prrt3 de39-339 mutant). We confirmed the membrane expression of the full-length form and the cleaved form of Prrt3 in oocytes by immunohistochemical staining using the anti-N-terminal antibody (Fig. 6A).

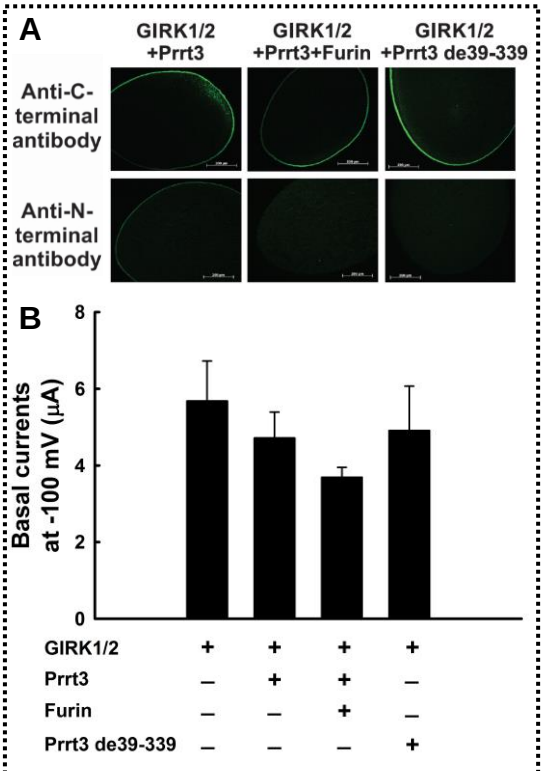

Figure 6. (A) Membrane expressions of the full-length form and the cleaved form of Prrt3 and (B) their basal GIRK current in oocytes.

By monitoring GIRK current, we observed that the full-length form Prrt3 shows similar basal activity with the cleaved form Prrt3 (Fig. 6B), suggesting that Prrt3 is not activated by the cleavage of its N-terminal extracellular domain.

(3) Identification of the physiological activator of Prrt3

In order to identify the physiological ligand of Prrt3, we performed ligand library screening by monitoring the GIRK current in oocytes expressing GIRK channel alone and GIRK channel with Prrt3. A small-molecule library (> 1000 compounds) was provided by Prof. Uesugi (Kyoto University). We have screened over 370 compounds and we observed that some of cholinergic ligands (including Oxo-M), but not ACh, slightly activates Prrt3 (Fig. 7). However, these compounds are not physiological ligands. We also observed that none of melatonin ligands, fatty acids and metabotropic glutamatergic ligands libraries induces response in Prrt3-expressing oocytes. Therefore, the physiological ligand of Prrt3 still remains unknown.

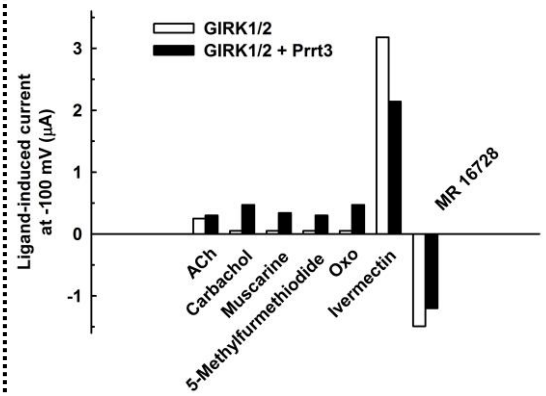

Figure 7. Results from the screening of cholinergic ligand library (including 72 compounds)

(4) Serendipitous finding by ligand library screening: a novel activator of GIRK channel

In the course of screening of the small-molecule library towards the identification of Prrt3 ligand by monitoring GIRK current, we happened to observe that Ivermectin (IVM), a modulator of a nicotinic ACh receptor (nAChR), activates GIRK channels even in the absence of the Prrt3. This suggests that IVM activates GIRK channel directly (Fig. 7). IVM is a widely used antiparasitic drug in humans and pets which activates glutamate-gated Cl^- (GluCl) channels in parasites. It is known that IVM binds to the transmembrane domains (TMs) of several ligand-gated channels, such as GluCl channels, glycine receptors, nAChRs and P2X receptors. We found that the GIRK channel, especially GIRK2 (Kir3.2), is activated by IVM directly in a $G_{\beta\gamma}$-independent manner, but the activation is dependent on phosphatidylinositol-4,5-biphosphate (PIP_2) (Fig. 8).

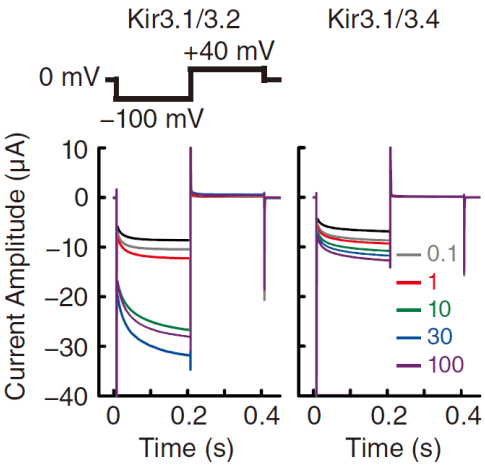

Figure 8. Characterization of IVM-induced GIRK current at different concentrations

We further identified a critical amino acid residue of GIRK2 for activation by IVM, Ile82, located in the slide helix between the TM1 and the N-terminal cytoplasmic tail domain (CTD) (Fig. 9).

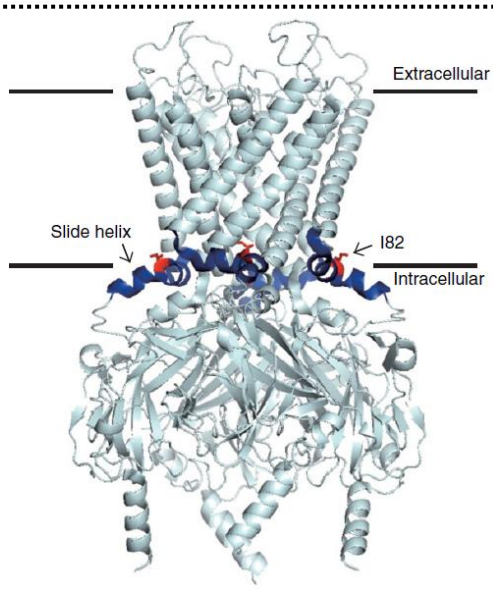

Figure 9. The structural determinant of GIRK2 for activation by IVM

The results demonstrate that the TM-CTD interface in GIRK channel, rather than the TMs, governs IVM-mediated activation and provide us with novel insights on the mode of action of IVM in ion channels.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　2　件）

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(2) I-Shan Chen, Michihiro Tateyama, Yuko Fukata, Motonari Uesugi, Yoshihiro Kubo.
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〔図書〕（計　0　件）

〔産業財産権〕

○出願状況（計　0　件）

○取得状況（計　0　件）

〔その他〕
ホームページ等

研究報告：抗寄生虫剤イベルメクチンによる GIRK チャネルの活性化機構の解明
http://www.nips.ac.jp/nips_research/2017/08/girk.html

６．研究組織
(1)研究代表者

陳　以珊（CHEN, I-Shan）
生理学研究所・分子細胞生理研究領域・特任助教
研究者番号：40757770

(2)研究分担者

None

(3)連携研究者

None

(4)研究協力者

久保　義弘（KUBO, Yoshihiro）
生理学研究所・教授

立山　充博（TATEYAMA, Michihiro）
生理学研究所・准教授

山本　友美（YAMAMOTO, Tomomi）
生理学研究所・技術職員

深田　優子（FUKATA, Yuko）
生理学研究所・准教授

上杉　志成（UESUGI, Motonari）
京都大学・教授