氏　　名	石井　裕
学位（専攻分野）	博士（理学）
学位記番号	総研大甲第1353号
学位授与の日付	平成２２年３月２４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	Identification and characterization of Cs^+-permeable K^+ channel current in the mouse cerebellar Purkinje cells evoked by molecular layer stimulation in lobules 9 and 10
論文審査委員	主　査　教授　井本　敬二 教授　鍋倉　淳一 教授　曽我部　正博（名古屋大学）

論文内容の要旨

The cerebellum consists of 10 lobules in mice which are distinguishable by their anatomical and functional differences. For example, lobule 10 is known to regulate the vestibular system. Purkinje cells (PCs) play critical roles in the cerebellar function as they are the only neuron type which send output from the cerebellum and as events of synaptic plasticity occur between parallel fibers and PCs. However, the differences of the synaptic currents of PCs between lobules have not been studied intensively so far. Thus, this study was started with the investigation of lobule-specific differences of the slow synaptic current.

Cerebellar parasagittal slices (250 μm) were obtained from young mice (postnatal days 14 - 18). PCs were identified by its large soma on the upright microscope using IR-DIC CCD camera. PCs in lobules 3, 9 and 10 were voltage-clamped at -70 mV by whole-cell patch clamp and slow synaptic currents evoked by a tetanic stimulation (100 Hz, 0.1 s) of the molecular layer (ML) were recorded.

Slow inward currents were observed in PCs similarly in lobules 3, 9 and 10. The metabotropic glutamate receptor 1 (mGluR1) is expressed in PCs and is known to activate two signaling cascades, G_q-pathway and "mGluR current", by a tetanic stimulation of parallel fibers in the ML. As expected, the recorded inward current was inhibited by mGluR1 antagonist, CPCCOEt (100 μM), suggesting that mGluR current was induced in all three lobules. In addition, a slow outward current was observed ahead of the inward current in lobules 9 and 10, but hardly in lobule 3. This lobule-specific outward current was not blocked by mGluR antagonists. As it has never been reported before, he aimed to identify the receptor(s) and the ion channel(s) underlying the current.

It is possible that the slow current is mediated by some types of metabotropic receptors and downstream signalings, but a P2 purinergic receptor antagonist, PPADS (10 μM) and an adenosine receptor antagonist, CGS15943 (10 μM) did not inhibit it. An intracellular Ca^{2+} chelator BAPTA (10 mM) did not affect it even 15 min after whole-cell configuration was achieved. Notably, it was significantly suppressed by a non-hydrolyzable GDP analog, GDP-βS (1 mM) in the pipette solution, demonstrating an involvement of G-protein pathway. Furthermore, the outward current was shown to be inhibited by metabotropic $GABA_B$ receptor antagonist, CGP55845 (1 μM), and the time to peak as well as the decay time course was decelerated by GABA uptake inhibitor. These results show that the slow outward current is mediated by $GABA_B$ receptor activation evoked by GABA released from inhibitory interneurons in the ML.

He investigated then the origin of the lobule-specific difference of the slow outward current focusing on three possible aspects, a cell density of

interneurons in the ML, presynaptic properties and an expression level of postsynaptic $GABA_B$ receptor. The density was estimated by using GAD67-GFP knock-in mice, whose GABAergic neurons are fluorescent. The cell density of interneurons in lobule 10 was significantly higher than that in lobule 3. The paired-pulse properties which reflect the presynaptic features were examined by recording $GABA_A$ receptor currents, but there was no significant difference between lobules. The $GABA_B$ receptor expression level analyzed by the outward current amplitude evoked by baclofen application was significantly higher in lobules 9 and 10 than in lobule 3. Finally, the relative current amplitudes between the $GABA_A$ receptor current by a single ML stimulation and the $GABA_B$ receptor current by a tetanic stimulation at the same position were compared in lobules 3 and 10. It was shown that $GABA_B$ current relative to $GABA_A$ one is significantly larger in lobule 10 than in lobule 3. Taken together, the differences of the cell density of interneurons in the ML and $GABA_B$ receptor expression level in PCs, at least partly, contribute to the difference of the slow outward current amplitude between lobules.

Next, he aimed to identify the molecular entity of the involved ion channel. As K^+ is the only ion to flow outward under the present recording condition, a sort of K^+ channel(s) was speculated to play a role. However, the outward current was inhibited neither by standard K^+ channel blockers, TEA (1 mM), Cs^+ (2 mM), nor by Ca^+-activated K^+ channel blockers Apamin (250 nM) (SK channel blocker), Penitrem A (1 μM), Paxilline (10 μM), Verruculogen (1 μM) (BK channel blockers). It is known that $GABA_B$ receptor activates G-protein coupled K^+ (GIRK) channel in the hippocampal CA1 pyramidal cells and in the cerebellar granule cells, but the $GABA_B$ receptor- mediated current in PC was not inhibited by tertiapin-Q, a selective blocker of GIRK channels. It is noteworthy that the outward Cs^+ current was recorded when a Cs^+-based intracellular solution was used, showing Cs^+ permeability of the channel.

One example of Cs^+ block insensitive- and Cs^+ permeable- K^+ channel is two-pore K^+ (KCNK) channel. Therefore, he looked for KCNK channels expressed in the mouse PCs by Allen Brain Atlas, an *in situ* hybridization database, and found that KCNK13 channel is highly expressed. KCNK13 channel cDNA was isolated from the cerebellum by RT-PCR and was characterized electrophysiologically in heterologous expression systems such as CHO-K1 cells and *Xenopus* oocytes, focusing on the Cs^+ permeability and the G-protein sensitivity.

KCNK13 channels expressed in CHO-K1 cells showed outward K^+ and also Cs^+ currents by whole-cell patch clamp when K^+- or Cs^+- based intracellular solution was used. Critical residues for Cs^+ block in the pore region are known for the plant voltage-sensitive K^+ channel KAT1 (Thr 259) and the inward rectifying K^+

channel Kir2.1 (Thr 141). In KCNK13 channel, Ser 109 and Ser 236 in the two pore regions were identified as equivalent residues by homology search, and a S109T & S236T double mutant was made. It showed K^+ outward current with the K^+-based intra-cellular solution but no Cs^+ current with the Cs^+-based one. These results show that KCNK13 channel is a candidate of Cs^+-permeable K^+ channel observed in PCs and that Ser 109 & Ser 236 define the Cs^+-permeability.

If KCNK13 channel can be activated upon $GABA_B$ receptor activation, a direct activation by G-protein is postulated as a possibility, like the case of GIRK channel by $G_{\beta\gamma}$. It was reported that some KCNK channels can be modulated by the downstream signaling of G-protein coupled receptors or by the direct binding of $G\alpha_q$, but there are no references describing the direct activation of KCNK channel by $G_{\beta\gamma}$. He, for the first time, observed in *Xenopus* oocytes that KCNK13 channel current increases by co-expression of $G_{\beta\gamma}$. By homology analyses of reported $G_{\beta\gamma}$ binding motifs, two motifs were identified in KCNK13 channel: one in the amino-terminal (EDNA) and the other is in the carboxy-terminal (SEMA) intracellular regions. In the motif-deleted mutants and mutants with all four residues mutated to Ala, the current enhancement by $G_{\beta\gamma}$ was not observed, showing that KCNK13 channel is a candidate effector of $GABA_B$ receptor via $G_{\beta\gamma}$ subunits.

In summary, he recorded slow synaptic outward currents through Cs^+ permeable K^+ channels in the mouse cerebellar Purkinje cells of lobules 9 and 10 upon a tetanic stimulation of the ML, and determined that they are mediated by $GABA_B$ receptor activation. Furthermore, he identified the two-pore K^+ channel KCNK13 as a legitimate candidate for the Cs^+-permeable K^+ channel activated by $GABA_B$ receptor via $G_{\beta\gamma}$ subunits in the Purkinje cells.

博士論文の審査結果の要旨

小脳は10個の小葉（lobule; L）から成り、それらの違いについて解剖学的・機能的な側面からこれまで多くの研究がなされている。しかし代謝型受容体を介する遅いシナプス電流（slow postsynaptic current; sPSC）の違いに着目した研究はない。申請者は、L3、L9、L10でプルキンエ細胞（PC）のsPSCを調べた結果、小脳分子層の高頻度刺激により、既知の内向きsPSCに加えて、これまでに報告のない一過性の外向きsPSCがL9とL10で惹起されることを見出した。本学位論文は、この外向きsPSCとそれを担うイオンチャネルの分子実体について検討したものである。

薬理学的な解析から、$GABA_B$受容体（$GABA_BR$）がこの外向きsPSCに関与していることが示された。さらに小葉間でのsPSCの違いについて実験を行ない、小脳分子層にあるGABA性の抑制性介在細胞の密度がL10でL3とL9より高いこと、baclofenにより$GABA_BR$を介して生じる電流量がL9、L10でL3よりも有意に大きいことを見出した。よって、これらの違いが少なくとも部分的に、小葉間での外向きsPSCの違いに関与している可能性が示された。

一般的に$GABA_BR$はG蛋白質共役型内向き整流性K^+チャネル（GIRK）を活性化するが、PCにはGIRKの主要サブユニットであるGIRK1が発現しておらず、外向きsPSCは選択的GIRK阻害剤であるtertiapin-Qによっては抑制されなかった。また外向きsPSCはCs^+で阻害されず、むしろCs^+透過性を示した。これらの結果より、外向きsPSCはGIRKにより担われているのではなく、他のK^+チャネルが活性化されている可能性が示唆された。

KCNKファミリーのK^+チャネルにはCs^+透過性を有するものがあり、PCではKCNK13が強く発現していることから、KCNK13の特性についての検討を、CHO-K1細胞および*Xenopus*卵母細胞の発現系を用いて行い、以下の結果を得た。KCNK13はCs^+透過性K^+チャネルであり、Ser109とSer236がCs^+透過性に重要なアミノ酸である。KCNK13電流は$G_{\beta\gamma}$の共発現によって電流が増強され、$G_{\beta\gamma}$が作用する細胞内モチーフ（EDNAとSEMA）の変異により、この電流増強効果が消失する。細胞外のCs^+によってGIRK電流は阻害されたが、KCNK13電流および$GABA_BR$電流は同濃度のCs^+で阻害されない。これらの実験結果より、KCNK13チャネルがPCにおいて$GABA_BR$によって活性化されるCs^+透過性K^+チャネルであることが強く示唆された。

遅いシナプス電流は主にG蛋白共役型受容体の活性化に続いて引き起こされる電流であり、様々な調節的役割を果たしていると考えられているが、その種類は多様であり、全体的な理解は不十分である。本論文は、PCで新たに外向きsPSCを発見し、その電流を担うK^+チャネルがCs^+透過性を有する特異なチャネルであり、KCNK13である可能性が高いことを示した。またKCNKチャネルが$G_{\beta\gamma}$により調節を受けるということも新しい発見である。従って、本論文は、小脳外向きsPSCの機能と分子的基盤に関する新しい知見を示したものであり、博士論文として価値の高いものと委員会全員一致で認めた。