氏　　名	清水　秀忠
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論文審査委員	主　査　教授　小林　悟 教授　野田　昌晴 教授　髙田　慎治 教授　門松　健治（名古屋大学）

論文内容の要旨

Sodium (Na) homeostasis is crucial for life and Na levels in body fluids are constantly monitored in the brain. The subfornical organ (SFO) is the center of the sensing responsible for the control of Na-intake behavior, where Na_x channels are expressed in specific glial cells as the Na-level sensor. Na_x channel is a concentration-sensitive Na channel with a threshold value of approximately 150 mM for the extracellular Na ion. The Na_x-positive glial cells are sensitive to an increase in the extracellular Na level in the physiological range, indicating that glial cells, not neurons, are the primary site of Na-level sensing. However, the mechanism by which the Na signal sensed by "inexcitable" glial cells is transferred to neurons has remained to be elucidated.

To gain insight into the cellular processes involving Na_x in glial cells, in this doctor thesis, He started in my study with screening for molecules interacting with Na_x using the yeast two-hybrid system with each of the cytoplasmic domains of mouse Na_x as bait. Among the positive clones isolated from a mouse DRG cDNA library by using the C-terminal region of Na_x as bait, three clones coded for the α subunit of Na^+/K^+-ATPase. A detailed analysis revealed that all these clones were identical and coded amino acid sequence of the region close to the cytoplasmic catalytic domain of the α1 subunit of Na^+/K^+-ATPase. The direct interaction between Na_x and the α1 subunit of Na^+/K^+-ATPase was verified by pull-down assays and the immunoprecipitation of the cell lysate.

Coexpression of the α1 subunit of Na^+/K^+-ATPase and Na_x channels was examined by double-fluorescent immunostaining using sections of the SFO and dissociated cells from the SFO. The α1 subunit was broadly distributed throughout the SFO, overlapping with the expression of Na_x channels. The confocal microscopic analyses with isolated cells from the SFO showed that both molecules were colocalized in the plasma membrane. Na_x channels were expressed in large round cells, but not in small cells with neurite-like processes, indicating that Na_x channels are expressed in glial cells including ependymal cells.

It is known that the α2 and α3 isoforms of Na^+/K^+-ATPase are also expressed in the brain. Experiments using the yeast two-hybrid system showed that the cytoplasmic fragment of α2 corresponding to the region of the α1 isoform isolated also interacted with the C-terminal region of Na_x, but that of α3 did not. Thus, Na_x has specific interaction with α1 and α2 isoforms of Na^+/K^+-ATPase. By *in situ* hybridization, he verified that the mRNAs encoding the α1 and α2 isoforms of Na^+/K^+-ATPase were expressed in the SFO with a similar pattern to the Na_x channels. However, signals for the α3 isoform were not detected in the SFO.

He speculated that Na_x may functionally regulate Na^+/K^+-ATPase through the close interaction between the two. To examine this idea *in vitro*, he established cell lines using C6 glioma cells in which the expression of *Na_x* channel is inducible under a

tetracycline-based system. He preferentially used one of the cell lines thus prepared, C6M16, for the following experiments. C6M16 cells showed significant Na^+ influx in response to an increase in the extracellular Na level within the physiological range (from 145 mM to 170 mM) specifically under the conditions where Na_x channels are expressed.

If the molecular properties of the Na^+/K^+-ATPase is changed by Na_x, cellular metabolism should be affected accordingly, because cells in the CNS use ~50% of their energy resources to drive the Na^+/K^+-ATPase activity. He then compared the cellular uptakes of a fluorescent glucose derivative (2-NBDG) in isotonic (145 mM) and hypertonic (170 mM) Na solutions. The C6M16 cells with Na_x expression showed approximately 1.6-fold greater activity for 2-NBDG uptake in the 170 mM Na solution as compared with in the 145 mM solution, while the uptake by the C6M16 cells without Na_x expression was not increased in the 170 mM Na solution. The increase in the uptake of 2-NBDG in Na_x-expressing cells in the 170 mM Na solution was completely inhibited by ouabain, a specific inhibitor of Na^+/K^+-ATPase.

Next, he tested the effect of overexpression of the Na_x-binding fragments of α 1 and α 2 subunits of Na^+/K^+-ATPase in C6M16 cells, because these fragments are expected to work as a competitor of Na^+/K^+-ATPase for binding to Na_x channels. As was expected, the transfection of an expression vector carrying the fragments of the α 1 and α 2 subunits significantly suppressed the metabolic response in the C6M16 cells with Na_x expression in the 170 mM Na solution. In contrast, overexpression of the fragment of the α 3 subunit, which was negative for interaction with Na_x channel, did not affect the metabolic activation.

The C-terminal fragment of Na_x was also expected to work as a competitor for the binding of Na_x channels to Na^+/K^+-ATPase. Unexpectedly but intriguingly, overexpression of the C-terminal fragment of Na_x further enhanced the 2-NBDG uptake, when it was coexpressed in Na_x-positive cells. This suggests that the C-terminal region of Na_x is also able to support Na^+/K^+-ATPase, as well as the native Na_x. However, the expression of the C-terminal fragment of Na_x by itself (without concomitant expression of the native Na_x) exerted no effect on the 2-NBDG uptake. This strongly suggests that a function of the native Na_x channel (presumably Na^+-influx activity) is also essential for the upregulation of the metabolic state, in addition to the function which is substitutable with the C-terminal region of Na_x.

To estimate the contribution of Na^+ influx itself to the metabolic activation, he tested the effect of the influx generated by a Na-specific ionophore, monensin, on the uptake of glucose. At a concentration of 0.5 μM, monensin triggered a small Na^+ influx into cells comparable to that of the C6M16 cells expressing Na_x when stimulated in the 170 mM solution. However, the application of 0.5 μM monensin to C6M16 cells without Na_x-channel expression did not enhance the 2-NBDG uptake, and higher concentrations of monensin were not effective either. In contrast, when

Na_x-expressing cells were treated with 0.5 μM and higher concentrations of monensin, the 2-NBDG uptake was markedly enhanced dose-dependently. These results clearly indicate that the increase of the Na-ion concentration in the cell is not enough by itself to trigger the uptake of glucose (metabolic stimulation), and that the presence of Na_x channel protein is required for the stimulation of glucose uptake by the cells. Importantly, the C-terminal fragment of Na_x induced markedly enhanced 2-NBDG uptake under the condition without Na_x-channel expression with 0.5 μM monensin. This indicates that the full-length Na_x channel can be replaced by the C-terminal fragment under the condition where Na^+ influx was secured by monensin. Taken together, it is probable that both pre-stimulation of Na^+/K^+-ATPase (by interaction with Na_x channels through the C-terminal region of Na_x) and Na^+ influx (through Na_x channels or monensin) are essential for the activation of the Na^+/K^+-ATPase and the cellular metabolic stimulation.

To examine whether the Na_x channel is indeed involved in the energy-control system in the Na_x-positive glial cells *in vivo*, he performed an imaging analysis of the uptake of glucose in the SFO using 2-NBDG. In the wild-type SFO, incubated with 2-NBDG in the 170 mM Na solution, an intensively labeled mesh-like structure became apparent, suggesting that fine glial processes in the SFO actively took up the fluorescent derivative of glucose. These results clearly indicate that the SFO tissue has activity to take up glucose in response to a Na-level increase, and the Na_x channel is an essential component for this mechanism.

Next, he examined the dissociated cells from the SFO of wild-type and Na_x-KO mice to confirm that the cells showing the enhancement of 2-NBDG uptake express Na_x channels. Only among the wild-type cells, cellular populations that intensively took up 2-NBDG in the 170 mM Na solution were present, and these cells were all positive for Na_x and GFAP. These results clearly indicate that the Na_x channel is an essential component for the upregulation of energy demand in the SFO under the high Na condition, as observed in the C6M16 cells. In support of this view, cells dissociated from the SFO of wild-type mice showed a markedly enhanced uptake of 2-NBDG in the presence of 0.5 μM monensin, while the same stimulation of the cells from *Na_x*-KO mice induced little enhancement of the uptake.

Increased demand for glucose by cells means that cellular glycolysis is enhanced to yield lactate (or pyruvate). To confirm this idea, he next measured the amounts of lactate and pyruvate released from the SFO as another parameter of the metabolic activity. The SFO tissues removed from mice of both genotypes were incubated in a modified Ringer solution (containing 145 mM or 160 mM Na) at 37°C for 30 hr. The wild-type SFO showed an increase in lactate secretion by ~60% compared with the *Na_x*-KO SFO, in 160 mM Na solution. On the other hand, amounts of pyruvate released into the medium were 10-fold lower than those of lactate and did not differ under the two different Na conditions. This indicates that anaerobic glycolysis

was stimulated in Na_x-positive glial cells of the SFO under the high Na condition.

Neurons in the SFO of *Na_x*-KO mice are hyperactivated under dehydrated conditions compared with wild-type mice. In the SFO, GABAergic neurons are one of the major neuronal types surrounded by Na_x-positive glial cells. Then he examined the neuronal activity of GABAergic neurons in the SFO using patch-clamp techniques in the cell-attached mode. He prepared acute slices containing the SFO from *GAD-GFP* mice and *GAD-GFP/Na_x*-KO, in which the GABAergic neurons bear enhanced green fluorescent protein (eGFP) as marker, and selected the eGFP-positive cells under a fluorescence microscope. The GABAergic neurons in the SFO of both wild-type and *Na_x*-KO mice showed spontaneous firing at a similar frequency (~4 Hz) under the 145 mM Na condition. After the extracellular Na-concentration was raised to 160 mM, the firing frequency of the GABAergic neurons in the SFO of wild-type mice gradually increased 2-fold, but that of *Na_x*-KO mice did not show a significant change.

Because metabolic activation leads to the release of lactate from Na_x-positive glial cells, he next checked the possibility that lactate mediates the signal from the glial cells to GABAergic neurons to control the SFO activity. When lactate was added at 1 mM to the perfusate, the firing frequency of GABAergic neurons in the SFO of both wild-type and *Na_x*-KO mice increased. Furthermore, when 1 mM of lactate was added under the high Na condition, no additive effect on the neuronal activity was observed. These results indicate that lactate and Na share a common pathway in the stimulation of GABAergic neurons in the SFO. Lactate was most effective at ~1 mM in promoting the firing rate, and at higher concentrations, the firing was rather suppressed.

The neural activation induced by the Na-level increase was inhibited by α-Cyano-4-hydroxycinnamic acid, an inhibitor of monocarboxylate transporters (MCTs). These results clearly indicate that the Na-dependent stimulation of GABAergic neurons is largely mediated by MCTs. He also examined the effect of the other metabolic monocarboxylates, pyruvate and acetate, both known to be transported by MCTs. When pyruvate was added at 1 mM to the perfusate, the firing frequency of GABAergic neurons in the SFO of both wild-type and *Na_x*-KO mice similarly increased. By contrast, when acetate was added at 1 mM to the perfusate, the firing frequency was not significantly changed in either genotype.

He further explored the activation mechanism underlying the increase in the firing rate of the GABAergic neurons. The finding that lactate and pyruvate are equally effective suggests that the GABAergic neurons are energetically stimulated. Moreover, he found that Na-dependent potentiation of the firing activity of the GABAergic neurons were reduced by diazoxide, an opener of the ATP-sensitive K channel (Kir6.2 / K_{ATP} channel): The K_{ATP} channel closes in response to the increase of intracellular ATP level and depolarizes the cell. So, he examined the membrane potential of the GABAergic neurons during the application of lactate or high Na solution. The membrane potential was depolarized by both lactate and Na, and the

depolarization effect was expectedly reduced by diazoxide. These data thus support the view that lactate serves as an energy substrate to up-regulate the firing activity of the GABAergic neurons.

From these results, the following cellular mechanism for the signaling from glial cells to neurons became clear. Na-level-dependent Na^+ influx through Na_x and direct interaction between Na_x and Na^+/K^+-ATPase are the basis for activation of Na^+/K^+-ATPase in the glial cells. Activation of Na^+/K^+-ATPase stimulates anaerobic metabolism of glucose by the glial cells, which produces lactate as the end product. There exist GABAergic neurons spontaneously firing in the SFO. Lactate released from the glial cells functions as the substance signaling to the neurons for activation. To my knowledge, this study is the first to show that glial cells take the initiative in the regulation of neural activity using lactate as a signaling substance.

論文の審査結果の要旨

申請者は、体液Naレベル恒常性の維持に関わる、塩分摂取行動の制御のための脳内Naレベル感知機構の詳細を解明した。Naレベルセンサー分子であるNa_xチャンネルは、細胞外Na濃度が150 mMを越えると開く性質があり、脳内の脳弓下器官(SFO)や終板脈管器官(OVLT)という感覚性脳室周囲器官に分布している。*Na_x*遺伝子ノックアウトマウス(*Na_x*-KOマウス)は、体液中のNaレベルが脱水によって上昇しても、それを感知できず塩分の摂取を回避しない。その後、Na_xは感覚性脳室周囲器官のグリア細胞（上衣細胞やアストロサイト）膜上に発現しており、その細胞膜はきつくニューロンを取り巻いていることが判明した。そこで、感覚細胞であるグリア細胞からニューロンに情報が伝達される仕組みの解明が主要な研究課題となっていた。

申請者は、まず、グリア細胞におけるNa_xの機能を探る手がかりを得るため、Na_xの細胞内領域に結合する分子を探索し、その結果、Na_xのC末端領域にNa^+/K^+-ATPaseの・サブユニットが結合することを見出した。この結合のNa^+/K^+-ATPaseに与える機能的影響を検討するため、蛍光グルコース誘導体(2-NBDG)の取り込みを指標にその活性を解析した。Na_xを発現する細胞では、細胞外Na濃度が生理的濃度から上昇するにつれてグルコース取り込み量が増加し、その増加はNa^+/K^+-ATPaseの特異的な機能阻害剤であるウワバインにより完全に抑えられることを示した。また、SFOから単離した細胞で、この活性化を示した細胞は、全てNa_xを発現するグリア細胞であることを明らかにした。さらに、Na_xのC末を介した結合が、Na^+/K^+-ATPaseの細胞内Naレベル上昇に応答したNa^+/K^+-ATPaseの活性化に必須であることを多くの実験によって証明した。また、解糖系の活性化は乳酸の産生・放出の亢進をもたらすと考えられるが、高Na条件下において、SFO組織からの乳酸放出量が、*Na_x*-KOマウスでは変化が無いのに対し、野生型マウスSFOでは亢進することを明らかにした。

申請者は、さらに、放出された乳酸の機能的役割について詳細な検討を進めた。SFOにはGABA作動性の抑制性ニューロンが多数存在し、それらはNa_x陽性のグリア細胞によって取り囲まれていることに着目し、SFOにおけるGABA作動性ニューロンの活動を電気生理学的に調べた結果、SFOのGABA作動性ニューロンは、生理的Na濃度条件下で約4 Hzで自発発火をしていることを発見した。細胞外Na濃度を160 mMまで上げると、野生型マウスの発火頻度は徐々に増加し約2倍に達したが、*Na_x*-KOマウスではこの応答が認められなかった。Na濃度を上げる代りに1ー2 mMの乳酸を加えると、野生型と*Na_x*-KOマウスのいずれにおいても発火頻度が増加した。さらに、野生型マウスの高Na条件における発火頻度上昇は、乳酸の細胞内輸送を担うモノカルボン酸輸送体の抑制剤、・-CHCAにより抑制されたことから、Na上昇シグナルの下流で乳酸が神経活動の制御に関与していることが明らかになった。以上、Na_xは、細胞外Naレベルの増加を感知して開口し、Na^+を流入させると共に、Na^+/K^+-ATPaseを活性化させること、その結果グリア細胞の解糖系が活性化し、乳酸の放出量が増加すること、乳酸はニューロンに取り込まれてその発火頻度を上昇させていることが明らかとなった。さらに、ニューロン内では、取り込んだ乳酸を代謝することによってATP濃度が上昇し、ATP感受性Kチャンネル(Kir6.2/K_{ATP}チャンネル)が閉口し、膜

電位上昇と発火頻度上昇が起きることを示した。

本研究は、これまで不明であった体液Naレベルの感知機構における、グリア細胞から神経細胞への情報伝達の仕組みを初めて明らかにしただけでなく、グリア細胞が自らのセンサーの情報に基づき神経活動を制御するという、グリア細胞の生理的重要性を示した画期的研究である。申請者の研究成果のもつ意義は極めて大きく、審査委員会は全員一致で学位論文として充分な内容を有するものと判定した。