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論文内容の要旨

The Na_x channel has been classified as part of a subfamily of voltage-gated sodium channels. The primary structure of Na_x, however, is markedly different from that of other voltage-gated sodium channels, including the key regions for voltage sensing and inactivation. Until now, the functional properties of the channel have been poorly understood, as attempts at functional expression of Na_x in heterologous systems have failed. In this thesis, the Na_x channel was revealed, for the first time, to be a new type of sodium channel that is sensitive to an increase in the extracellular sodium concentration.

Na_x-deficient mice, in which the *Na_x* gene was knocked-out by insertion of the *lacZ* gene in-frame, showed that the Na_x channel was expressed in neurons in the circum ventricular organs (CVOs), like the subfornical organ (SFO) and organum vasculosum laminae terminalis (OVLT), regions important for control of the body fluid ionic balance. Under conditions of thirst, *Na_x*-deficient mice showed hyperactivity of neurons in these areas as compared with wild-type mice. Cells in CVOs are able to monitor body fluid conditions directly, because such regions lack a blood-brain barrier. Under parched conditions, the sodium concentration and plasma osmolarity increase by 5–10%. In this state, wild-type mice stopped ingesting salt. In contrast, *Na_x*-deficient mice did not. Thus, Na_x expressed in the SFO and OVLT is expected to play an essential role in sodium-level sensing in the brain.

To verify the role of Na_x, the SFO and OVLT were directly stimulated with hypertonic sodium solutions by intracerebroventricular (ICV) infusion and the preference for a 0.3 M NaCl solution over pure water was examined by a two-bottle test. When a hypertonic sodium solution was infused, wild-type mice avoided salt-intake. In contrast, *Na_x*-deficient mice showed no such aversion. These results indicate that Na_x is involved in sodium-level sensing in the brain.

Cellular responses of Na_x were examined next by imaging analysis using an indicator for sodium ion. The analysis was performed first using dorsal root ganglion (DRG) neurons because all are Na_x-positive, whereas only a subpopulation of neurons in the SFO or OVLT are Na_x-positive. When $[Na^+]_o$ was increased from the control amount of 145 mM (control sodium solution) to 170 mM (high sodium solution) by bath application of an NaCl solution, the $[Na^+]_i$ of dissociated neurons derived from wild-type mice showed a pronounced increase. In contrast, no significant increase in the $[Na^+]_i$ was detected in DRG neurons isolated from *Na_x*-deficient mice. None of the cells (from either wild-type or *Na_x*-deficient mice) responded to the rise in osmolarity or chloride concentration $[Cl^-]_o$ to that of the 'high sodium solution'. When the sodium concentration was raised with sodium methanesulfonate, on the other hand, there occurred a response. Thus the neurons responded to the rise in $[Na^+]_o$ but not to the rise in osmolarity or $[Cl^-]_o$. $[Na^+]_o$ at the half maximal value ($C_{1/2}$) was determined to be 159 mM from the relationship between the $[Na^+]_i$ increase rate and $[Na^+]_o$. When $[Na^+]_o$ was lowered from the control amount of 145 mM, no $[Na^+]_i$ response was seen. The cytosolic sodium response was observed in DRG neurons of various sizes from wild-type mice, consistent with the ubiquitous expression of Na_x in DRG neurons, and tetrodotoxin (TTX)—a potent blocker of TTX-sensitive

voltage-gated sodium channels—did not antagonize the response.

This change in $[Na^+]_i$ was speculated to be a result of sodium inflow from extracellular space. This possibility was verified by whole-cell current recordings of DRG neurons using a patch-clamp technique. When 'high sodium solution' was applied to cells derived from wild-type mice, inward currents were observed. The current amplitude was consistent with that estimated from the ion-imaging studies. By contrast, the current was not observed in cells derived from Na_x-deficient mice, and it was not inactivated under 'high sodium solution' conditions; instead, it disappeared rapidly when the amount of extracellular sodium returned to normal. There was no voltage dependency during the application of 'high sodium solution' and the current amplitude was not affected by TTX.

For further confirmation, an Na_x expression vector was constructed using mouse Na_x cDNA and introduced into the dissociated DRG neurons from *Na_x*-deficient mice. When $[Na^+]_o$ was increased from 145 mM to 170 mM, an $[Na^+]_i$ response similar to that in wild-type neurons appeared. In all control experiments of mock transfection, no $[Na^+]_i$ response was detected. It was thus revealed that Na_x is a newly identified type of sodium channel that is sensitive to an increase in the extracellular sodium concentration.

Finally, the same experiments were carried out on SFO cells. A similar $[Na^+]_i$ response was detected in neurons that were dissociated from the SFO of wild-type mice, and all the $[Na^+]_i$–responsive cells in this region were also Na_x-immunoreactive. When 'high sodium solution' was applied to the cells, the $[Na^+]_i$ of these neurons increased over a time-course similar to that in DRG cells. The Na_x-immunopositive SFO neurons responded to an increase in $[Na^+]_o$, but not to increases in osmolarity or $[Cl^-]_o$. The $[Na^+]_o$ at half maximal value ($C_{1/2}$) was approximately equal to the concentration obtained from DRG. In contrast, SFO neurons derived from *Na_x*-deficient mice did not show such responses. When the expression vector for mouse Na_x cDNA was introduced *in vitro* into SFO neurons from *Na_x*-deficient mice, the sodium sensitivity of the neuron was restored. These findings indicate that the Na_x channel is a sodium receptor in the brain.

In order to identify the neurons which express Na_x in SFO, the localization of Na_x in SFO was examined by immunostaining. Most immunopositive neurons were observed in the peripheral region of SFO and overlapped with GABA-immunopositive neurons. Furthermore, all of the GABA-immunopositive neurons isolated from SFO responded to the increase in the extracellular sodium level. Based on these findings, a locking hypothesis in SFO for the regulation of salt-intake behavior was proposed: the GABAergic inhibitory neurons which express Na_x lock the activity of the neurons that continuously stimulate the salt-intake behavior of animals under certain conditions, when the sodium level of body fluids rises.

Finally, the localization of Na_x throughout the visceral organs at the cellular level was examined using a specific antibody against Na_x in wild-type mice and lacZ expression in the gene-targeted mice in which the *Na_x* gene is replaced with the *lacZ* gene. In visceral organs including the lung, heart, intestine, bladder, kidney and tongue, a subset of Schwann cells within the peripheral nerve trunks were highly positive for Na_x. An electron microscopic analysis indicated that these Na_x-

positive cells were non-myelinating Schwann cells. In addition, Na_x-positive signals were observed in the alveolar type II cells in the lung, which actively absorb sodium and water to help the efficient gas exchange through the alveolar surface. Taken together, the Na_x channel appears to be involved in several important functions through its peculiar properties not only in the CNS but also in the peripheral organs.

論文の審査結果の要旨

Na_x チャンネルは長い間機能不明のチャンネル分子であったが、最近の Na_x 遺伝子欠損マウスの解析から、脳内で Na_x は塩濃度検出器官と言われる脳弓下器官(SFO)と終板脈管器官(OVLT)の神経細胞に発現し、欠損マウスは塩分摂取行動に異常が認められることから、Na_x が脳におけるナトリウム濃度検出に重要な役割を果たすと予想された。しかしながらその分子メカニズムについては全くわかっていなかった。

申請者はまずナトリウムイオン感受性色素を使用して細胞内に流入するナトリウム測定することにより、Na_x の特性を検討した。後根神経節(DRG)細胞とSFO の神経細胞を用いて、細胞外ナトリウムイオン濃度($[Na^+]_o$)を 145 mM から 170 mM に増加させた時、野生型マウス由来の細胞では、細胞内ナトリウムイオン濃度($[Na^+]_i$)の顕著な増加が見られた。一方、Na_x 遺伝子欠損マウスから単離した細胞ではまったくみられなかった。パッチクランプ技術を用いて野生型マウス由来の DRG 細胞のホールセル電流記録を行い、これに対応する内向き電流を観察した。この電流は不活化せず、細胞外のナトリウム濃度が正常に戻った時、初めて急速に消失した。さらにマウス Na_x cDNA を使用して Na_x 発現ベクターを構築し、Na_x 遺伝子欠損マウスから分離した神経細胞に導入した。 その結果、遺伝子導入された細胞から上記の$[Na^+]_i$ 上昇応答が観察された。以上の知見から Na_x が細胞外のナトリウム濃度の増加に感受性のある、新しいタイプのナトリウムチャンネルであることが明らかとなった。

次に申請者は中枢神経系での Na_x の機能を調べた。脳室内微量注入法により高張ナトリウム溶液で SFO および OVLT の直接刺激を行い、二瓶法により塩分摂取行動を観察した。高張ナトリウム溶液を注入すると野生型マウスは塩分摂取を直ちに回避したが、Na_x 遺伝子欠損マウスは回避行動をとらず、Na_x が脳内ナトリウム濃度検出に重要であることが判明した。また、SFO において Na_x の局在を免疫染色によって調べたところ、GABA 免疫陽性神経細胞で陽性であることが判明した。更に、SFO から分離された GABA 免疫陽性神経細胞はすべて細胞外のナトリウム濃度の増加に応答することを確認した。これらの知見に基づき、申請者は SFO における塩分摂取行動制御機構としてロッキング仮説を立案した。体液のナトリウム濃度が上昇すると、Na_x を発現する GABA 作動性抑制性神経細胞が活性化し、動物の塩分摂取行動を連続的に刺激している神経細胞の活動を停止するというものである。

さらに申請者は内臓全体にわたる Na_x の局在を細胞レベルで解析した。免疫染色した切片の光学顕微鏡、電子顕微鏡による解析から、肺、心臓、腸、膀胱、腎臓および舌を含む内臓の器官では、髄鞘形成しないシュワン細胞に発現することを明らかにした。さらに、肺では肺胞 II 型細胞に発現することを見い出した。以上より Na_x チャンネルは、中枢神経系だけでなく末梢器官中においても、その特有の特性によってナトリウムバランスに関するいくつかの重要な生理機能を担うことが示唆された。

本研究は、これまで機能の不明であった Na_x チャンネルが細胞外ナトリウム濃度の増加を感知して開口する新たなタイプのナトリウムチャンネルであることを初めて明らかにしたものであり、申請者の研究成果の学問的意義は極めて深く、今後の当該分野の研究に新展開をもたらすものであり、審査委員会は全員一致で学位論文として十分であると判定した

学位審査会では、先ず、30 分間申請者に学位論文の内容について発表してもらい、その後、質疑応答を行ったが、関連する周辺領域の知識は十分であり、質問に対する応答も的確であった。申請者は既に、内容の一部を原著論文として一流国際誌に発表済みであり、学位論文も英語で書

かれていることから、英語の能力も十分であると判断した。審査の結果、本学位申請論文は、学位授与にふさわしい水準に達していると判定した。