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論文内容の要旨

The maxi-anion channel is a voltage-dependent, large-conductance anion selective channel. The maxi-anion channels express in a large variety of cell types. Roles of the maxi-anion channel in cell volume regulation, programmed cell death, ischemia and purinergic signaling have been well documented. Its main physiological function is considered as a pathway for the regulated release of ATP and glutamate. The maxi-anion channel has been extensively characterized with respect to biophysical and pharmacological properties, although its molecular identity is unknown. In spite of some progress in recent years, the mechanisms by which maxi-anion channel is regulated remain incompletely understood. Hence, the purpose of his study was to reveal the activation mechanisms of the maxi-anion channel. Also, He tested a hypothesis of TTHY1 as a molecular identity of the maxi-anion channel.

In the present work, He studied excision-induced activation maxi-anion channel by patch-clamp in the inside-out mode. He demonstrated that excision-activated maxi-anion channels in mammary C127 cells show similar biophysical profiles in artificially designed intracellular solution as previously reported for this cell line under different conditions. The biophysical profile includes a large single-channel conductance of ~365 pS, a linear current-to-voltage relationship, time- and voltage-dependent inactivation at higher voltages and anionic selectivity. Addition of the MgATP into the intracellular solution at a physiological level (1 mM) completely abolished excision-induced activation of the maxi-anion channel in inside-out patches. In contrast, the non-hydrolysable analog of ATP, ANP-PNP, failed to suppress the maxi-anion channel activation. Based on these results, He supposed that the excised membrane patches retained auxiliary proteins including kinases and phosphatases, and that under ATP-free conditions, phosphatase activities dominated and caused the channel opening whereas in the presence of Mg-ATP the channel protein remained phosphorylated and stayed in the inactive closed state. Therefore, He next addressed the following question: what kind of phosphatase is involved in excision-induced activation of the maxi-anion channel? First He tested the effects of Ser/Thr phosphatase inhibitors (10-100 nM okadaic acid, 1 μM cyclosporine A and 1 μM FK520) on excision-induced activation of the maxi-anion channel. However, neither one had any significant effect on the channel activation. In contrast, broad-spectrum tyrosine phosphatase inhibitors (TPhIC 1%; 1 mM orthovanadate; 0.1 mM dephostatin and 0.1 mM *p*-bromoteramisole) markedly suppressed the maxi-anion channel activation. These results strongly suggest an involvement of some protein tyrosine, rather than Ser/Thr, phosphatase in the maxi-anion channel activation. In order to strengthen this inference on the role of tyrosine phosphorylation/dephosphorylation process in the maxi-anion channel activation, He next performed the experiments using inhibitors of protein kinases. The rationale was that in the experimental conditions favoring the phosphorylation process, protein kinase inhibitors should be able to restore the channel activity from its inactive state. In the presence of 1 mM MgATP, broad-spectrum serine/treonine kinase inhibitor, H7, had no significant effect on the channel activation, but, in contrast, two tyrosine kinase inhibitors, AG18 and genestein, caused robust activation of the maxi-anion channel over in the presence of MgATP. These results provide independent evidence that tyrosine, but not Ser/Thr, phosphorylation is involved in the inhibition of the maxi-anion channel in C127 cells.

Which protein tyrosine phosphatase (PTP) is responsible for the maxi-anion channel activation? To identify the specific type of PTP, He tested available inhibitors specific to particular types of PTP such as NSC 95397, PTP inhibitor IV, CD45 inhibitor, PTP inhibitor II and bpV (bipy). However, there were no statistically significant effects of either one on the maxi-anion channel activation. He could not test all types of PTP due to limited availability of the selective inhibitors. He adopted molecular biological approaches in order to identify the maxi-anion channel-specific PTP. He supposed that receptor tyrosine phosphatases (RPTP) could be better candidates, because they are membrane proteins and would be likely retained in the membrane patches after excision. RPTPζ is one of such phosphatases that are known to be involved in membrane protein dephosphorylation. The mRNA for RPTPζ protein was found in both C127 cells and in cells isolated from the mouse adult fibroblasts (MAF) by the RT-PCR analysis. To test an involvement of RPTPζ in the maxi-anion channel activation, He prepared primary cultures of MAFs from wild-type (WT) and RPTPζ-knockout (RPTPζ-KO) mice. Membrane patches derived from WT MAFs responded to patch excision with robust activation of maxi-anion channels, which showed properties similar to those observed in C127 cells. When He examined the excision-induced maxi-anion channel activation, He found that the channel activation rate was significantly slower in RPTPζ-KO MAFs compared to MAFs from WT mice. Transfection of wild-type, but not the dominant-negative (dn) mutant, RPTPζ into RPTPζ-KO MAFs recovered the maxi-anion channel activation rate to the level comparable to that of the WT MAFs. These results strongly suggest that RPTPζ represents an important part of the excision-induced activation mechanism of the maxi-anion channel. It should be noted, however, that the maxi-anion channel current was not completely eliminated in RPTPζ-KO MAFs. He supposes that some other type(s) of phosphatase could also be involved in dephosphorylation of the maxi-anion channel protein upon membrane patch excision.

Next, He used cell-attached mode of patch-clamp in order to retain the integrity of the cells. Cell swelling in response to hypotonic stimulation is known to activate maxi-anion channels. Indeed, in cell-attached experiment, the activation of the maxi-anion channel occurred after approximately 15 min of application of the hypotonic solution. He examined the effect of vanadate on the hypotonicity-induced maxi-anion channel activation. After 10-min pretreatment with vanadate, the hypotonicity-induced maxi-anion channel activation was significantly suppressed. This result suggests that tyrosine dephosphorylation play a role in hypotonicity-induced activation of the maxi-anion channel in intact cells.

The maxi-anion channel has been shown to represent a major ATP-conductive pathway mediating osmotic swelling-induced release of ATP from C127 cells (Sabirov et al. 2001 J. Gen. Physiol. 118, 251-266). Thus, He expected that some of the drugs above tested would affect swelling-induced ATP release in a way consistent with their modulatory effects on activation of the maxi-anion channel found in isolated membrane patches. Broad-spectrum PTP inhibitors, dephostatin (0.1 mM) and *p*-bromotetramisol (0.1 mM) effectively suppressed hypotonicity-induced ATP release from C127 cells. These results are consistent with inhibitory effects of these drugs on the maxi-anion channel activation observed in the present patch-clamp experiments. However, orthovanadate had no effect on the ATP release when used at 1 mM, and even enhanced hypotonicity-induced ATP release at higher

concentrations. He interprets this result by effects of this non-specific drug on some other processes involved in hypotonicity-induced ATP release, possibly exocytosis or some other pathways. Consistent with the present patch-clamp data, a broad-spectrum Ser/Thr kinase inhibitor, H7, had no significant effect on swelling-induced release of ATP. In contrast, a tyrosine kinase inhibitor, genistein, but not its inactive analog, daidzein, had a stimulatory effect on ATP release upon hypotonic stimulation. These results parallel the enhancing effect of genistein on the maxi-anion channel activation in excised inside-out patches. Another non-specific tyrosine kinase inhibitor, AG18, had no significant effect on swelling-induced ATP release being in contrast to its enhancing effect of the maxi-anion channel activation. He suppose that the activating effect of this drug on the maxi-anion channel seen in the experiments with excised inside-out patches was masked by its action on some other systems involved in swelling-induced ATP release from intact C127 cells. Next He compared swelling-induced ATP release between WT and RPTPζ-KO MAFs. In these experiments, time-dependent ATP release from swollen MAFs derived from RPTPζ-KO mice was significantly lower compared to that from WT. This result suggests that protein phosphatase RPTPζ is involve in the swelling-induced activation of maxi-anion channels or swelling-induced ATP release.

In the present study, He also provided firm evidence for divalent cation-dependent activation of the maxi-anion channel. Although maxi-anion channels can be activated even in the absence of divalent cations, an increase in the concentrations of free Ca^{2+} or Mg^{2+} greatly accelerated the excision-induced maxi-anion channel activation in a time- and concentration-dependent manner. The mechanism of Ca^{2+} effect on the activation of the maxi-anion channel may involve a Ca^{2+}-dependent protein phosphatase. Therefore, He next tested the effect of high intracellular free Ca^{2+} on the maxi-anion channel activation in the presence of 1 mM MgATP. Under these conditions, 1 μM free [Ca^{2+}] was able to overcome the inhibitory effect of MgATP on the maxi-anion channel. The rescuing effect of high Ca^{2+}-induced channel activation was significantly suppressed by exposure to 1 mM vanadate. In contrast, the calcineurin inhibitor, compound FK520 did not significantly alter the Ca^{2+}-induced activation of the maxi-anion channel. Since RPTPζ activity is Ca^{2+}-independent (T. Shintani and M. Noda: personal communication), our results may suggest an involvement of a Ca^{2+}-dependent tyrosine phosphatase other than RPTPζ in the Ca^{2+}-dependent activation of the channel.

Albeit important general biological functions, the molecular entity of the maxi-anion channel has not yet been identified. Recently, Suzuki and Mizuno (Suzuki and Mizuno, 2004. J. Biol. Chem. 279, 22461-22468) have reported that a gene *tweety* found in *Drosophila flightless* locus has a structure similar to those of known channels. The human homologs of *tweety* (hTTYH1-3) have been suggested to provide the product, which represents a novel large-conductance Ca^{2+}-activated chloride channel, while a related gene hTTYH1 gave rise to functional expression of the swelling-activated chloride channel. It has been hypothesized that hTTYH1 might be the large-conductance Ca^{2+}-activated chloride channel (Suzuki, 2006. Exp. Physiol. 91, 141-147). In order to test this attractive hypothesis, He first searched the cells completely lacking the maxi-anion channel activity. Among several cell lines tested, only the HEK293T cell line was found to exhibit no activity of maxi-anion channels when tested in conditions favoring the channel opening in the excised inside-out mode. Next, He transfected two splice variants of the TTYH1 clone (TTYH1-E and TTYH1-SV) into HEK293T cells and assayed the

maxi-anion channel activity on 1-5 days after transfection. The TTYH1-E-transfected cells never showed time-dependent rise of membrane conductance up to about 20 min of patch excision when tested by applying +25 mV test pulses. However, patch currents abruptly exhibited somewhat noisy behavior with not well-defined single-channel amplitudes, over 20 min after patch excision. Very similar results were obtained in TTYH1-SV-transfected cells as well. Moreover, WT HEK293T cells also failed to respond to patch excision with activation of distinct single-channel events, although seal breakdown-like noisy currents often appeared over 20 min after excision. Importantly, any typical maxi-anion channel activity with a standard amplitude of ~10 pA at +25mV and its time-dependent inactivation at higher positive and negative voltages could never be observed in either nonetrasfected HEK293T cells or those transfected with TTYH1-E or TTYH1-SV clones. The data did not positively support an idea that the two clones tested alone represent the maxi-anion channel of the phenotype observed in C127 cells.

論文の審査結果の要旨

Maxi-anionチャネルは、大きなコンダクタンスを特徴とする陰イオン選択性チャネルで、種々の細胞に発現し、細胞容積調節や細胞死等において、また、ATP やグルタミン酸の放出路として、役割を果たすことが知られている。本論文に於いて、申請者 Toychiev 氏は、まだ知られていないこのチャネルの活性化機構の解明に取り組んだ。

申請者は、まず、細胞からパッチ膜を引き抜くとチャネルの活性化が見られること、ATP 存在下では活性化がみられないことから、リン酸化による抑制と脱リン酸化による活性化を想定した。この仮説の下に、薬理学的実験を行い、orthovanadate などのチロシン脱リン酸化酵素の阻害薬によって活性化が阻害されること、genestein などのチロシンリン酸化酵素の阻害薬によって ATP 存在下でも活性化が起こることを観察した。

次のステップとして、申請者は、役割を果たすチロシン脱リン酸化酵素の中で、膜結合型の RPTP・ に着目した。線維芽細胞において、RT-PCR 法により、RNA の発現が確認され、RPTP・・のノックアウトマウスの線維芽細胞ではパッチ膜の引き抜きによるチャネル活性化のレベルが低く、また、そこに、RPTP・・を transfection することにより活性化のレベルが高まるため、RPTP・・が重要な役割を果たしていることが示された。

さらに、申請者は、Maxi-anion チャネルの活性化に伴う重要な細胞生理学的現象である、低浸透圧刺激による ATP の放出についても解析し、薬剤の副作用のためか、チャネル活性化のデータと若干の相違はあるものの、ほぼ、上述のシナリオに沿う結果を得た。

このように、本研究において、申請者 Toychiev 氏は、Maxi-anion チャネルの活性化機構について、自身のひとつひとつの現象観察に基づいて、次のステップの実験を緻密に計画、遂行することにより、膜結合型チロシン脱リン酸化酵素による脱リン酸化が重要な役割を果たしていることを証明し、さらに、低浸透圧応答という細胞現象にまで展開した。この点において、本研究は、非常に大きな意義を持つと判断される。

申請者は、もうひとつの活性化機構として知られる細胞内 Ca^{2+}, Mg^{2+} による活性化についても薬理学的解析を行い、Vanadate 感受性を持つ Ca^{2+} 依存性のチロシン脱リン酸化酵素が関与する可能性を示した。さらに、Maxi-anion チャネルの分子実体としての可能性が想定されている TTYH1 分子を、Max- anion チャネルを発現していない HEK 細胞に発現させて機能解析を行い、典型的な Maxi-anion

チャネルの活性化は観察されなかったというデータを得て、現時点で、この分子が Maxi- anion チャネルの分子実体であることが積極的には支持されないことを示した。この部分についても、混沌とした状況において、明確なデータを提示することにより、今後のこの研究分野に指針を与えるという意義があると考えられる。

以上のように、本研究は、重要な生理学的意義を持ち、また、今後のさらなる発展が強く期待できる優れた研究である。よって、審査委員会は、全員一致で、本論文が学位論文として相応しいものであると判断した。