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研究室で撮影した本人のスナップ写真、及び発表論文のコピーを添付

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研究テーマ 心血管細胞におけるイオン代謝の分子機序の研究

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表 (有)・無 (学会名・内容)

第71回日本生化学会大会

Na^+/H^+ exchanger (NHE_1) におけるカルシニューリン類似タンパク質の結合部位とその役割

(2) 学会誌等に発表した論文 有・(無) (雑誌名・論文名)

3．今後の研究計画

心筋細胞膜 Na^+/H^+交換(NHE1)輸送体の分子内機能ドメインを明らかにするための分子生物学の研究に継続進めし、この輸送体にする心筋細胞膜内 pH 及増殖因子や高浸透圧制御の機序及び病態との関連を明らかにする。 Na^+/H^+交換(NHE1)輸送体蛋白質の細胞質ドメインの活性調節に、詳しい解析を行っている。

4．研究指導者の意見

パン・テンシアンさんは、現在循環器病センターで Na^+/H^+交換輸送体(NHE)の構造と機能、特にカルシニューリン類似タンパク質(CHP)とNHE1との相互作用に関する研究を行っています。DNA、タンパク質、細胞等の基本的な扱いをトレーニングした後、現在では十分一人で実験を進めていけるまで成長しました。彼は熱心に注意深く実験を行う点で大変優れており、彼が堅実な成果を収めてきたのもその彼の誠実な性格がいい方に作用したものと考えています。初めのうちはコミュニケーションがうまくいかなかったこともありましたが、最近彼の日本語と英語の能力もかなり上達し、日常会話ではあまり苦労しないようになりました。この調子でさらに飛躍するよう願っている次第です。

研究指導者氏名 重川宗一 (重川印)

5．研究報告

別紙形式を参考に、報告本文4000字以上で報告して下さい（枚数自由・ワープロ使用）

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

論文発表に当っては、日中医学協会－日本財団補助金による旨を明記して下さい。

研究テーマ：Na^+/H^+交換(NHE1)におけるカルシニェーリン類似蛋白質の結合部位とその役割

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要旨：

細胞形質膜に普遍的に発現する Na^+/H^+交換(NHE1)は、増殖因子や高浸透圧等、様々な刺激によって活性化されることが知られる。活性化の全貌はまだ明らかでないが、NHE1の細胞質ドメインとカルモデェリンやその他の制御因子との相互作用が重要と考えられている。NHE1 に結合する蛋白質としてカルシニューリン類似蛋白質(CHP)が最近クローニングされたが(Lin X, 1996;93:12631-12636)、その NHE1 における正確な結合部位と活性調節における役割には疑問が残された。私たちは、CHP 抗体と NHE1 抗体、NHE1 の様々な MBP 融合蛋白質、CHP の様々な GST 融合蛋白質作成し、in vitro オーバーレイアツセイ、共免疫沈降法によって、NHE1 と CHP との相互作用検討した。CHP の様々な GFP 融合蛋白質作成し、細胞に NHE1 と GFP-CHP との相互作用検討した。その結果、NHE1 細胞質ドメイン中の膜貫通ドメインに近いアミノ酸 510-540 が CHP 結合部位同定された。この領域は、以前報告されたもの(aa566-635)とは完全に異なる。私たちは最終目的に、細胞に発現した変異蛋白質 NHE1 delat 515-530(aa515-530 を欠失)と NHE1 I522C/D528C/H529C と、CHP との結合が検出できない程弱いことを確認した。また免疫染色の結果、この変異蛋白質 NHE1 I522C/D528C/H529C の大部分は細胞内膜系(Golgi ?)にとどまっており、NHE1 の膜へのターゲティングにおける CHP の関係が想定される。細胞に NHE1 と GFP-CHP との相互結合が高浸透圧の調節を確認した。しかし一方で、この領域は私たちが以前報告した pHi 感受性維持、ATP 感受性、増殖因子活性化等に関与するサブドメイン I (aa503-595)の中にあり、CHP が NHE1 の活性調節に関与する可能性もあり、現在より詳しい解析を行っている。

Interaction of the Na^+/H^+ exchanger (NHE1) with Calcineurin-homologue Protein (CHP): CHP Binding Domain and its Physiological Role

KEY WORDS

Na^+-H^+ exchanger, calcineurin B homologue protein, protein-protein interaction, intracellular pH

The abbreviation used are

NHE1, Na^+/H^+ exchanger isoform 1; CHP, calcineurin B homologous protein; EIPA, 5-(N-ethyl-N isopropyl)amiloride; GFP, green fluorescent protein; GST, glutathione S-transferase; MBP, maltose binding protein; pH_i, intracellular pH; pH_o, extracellular pH; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; aa, amino acid.

Purpose (目的)

The Na^+/H^+ exchanger (NHE) is an important regulator for intracellular pH (pH_i), cell volume, and transepithelial Na^+ transport (1-3). It exists in virtually all cells with cell type-dependent pattern of isoform expression. Among six known isoforms of NHE, a ubiquitous isoform NHE1 is known to be rapidly activated in response to a variety of extracellular stimuli ranging from growth factors to mechanical stimuli such as hyperosmotic stress and cell spreading (4-6). Some accessory regulatory factors has been suggested to be involved in the regulation of NHE1. Calmodulin is the first identified regulatory factor that is involved in Ca^{2+}-induced activation of NHE1 (7-9). Recently, the calcineurin B homologous protein (CHP) has been cloned as a candidate for such regulatory factors of NHE1 (10). However, the CHP-binding domain within NHE1 has not yet been precisely identified in that report (10). In addition, evidence presented in that study (10) was not enough to understand completely a regulatory role of CHP. In this study, we tried to identify precisely the CHP-binding site within NHE1 and to clarify the role of CHP by analyzing the mutant NHEs that can not bind CHP. The data suggest that CHP may be involved in protein processing rather than the acute regulation of NHE1.

EXPERIMENTAL PROCEDURES (方法)

Antibodies. The NHE1-specific polyclonal antibody was purified from rabbits immunized with maltose-binding protein (MBP) fusion protein containing the cytoplasmic domain (aa 503-815) of NHE1 (7). The CHP-specific polyclonal antibody (IgG fraction) was purified from rabbits immunized with GST/CHP 1-195 fusion protein.

Plasmid construction and purification of fusion proteins. All the plasmids used in this study were constructed based on production of DNA fragments by polymerase chain reaction (PCR), ligation into various cloning vectors and automated sequencing of the inserted fragment. Plasmids were transformed into *E.coli* (HB 101) for expression of the fusion proteins or plasmid preparation. MBP or GST fusion proteins were purified according to manufacturar's protocol.

Cells and plasmid transfection. The Na^+/H^+ antiporter-deficient PS 120 cells and transfectants were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM $NaHCO_3$ and supplemented with 7.5% (v/v) fetal calf serum, penicillin 50 units/ ml and streptomycin 50 mg/L in the presence of 5% CO_2. Cells ($5x10^5$ cells/10 cm dish) were transfected with wild type and various mutant NHE1 plasmids or GFP-CHP plasmids (20 μg) by calcium-phosphate co-precipitation technique. Cell populations that stably express NHE1 variants were selected by the "H^+-killing"(11), and cells stably expressing GFP variants were selected with G418.

Immunoblot and immunoprecipitation. For immunoprecipitaion, cells were harvested in a lysis buffer [containing: 150 mM NaCl, 20 mM Hepes/Tris (pH7.4), 1% Triton-100, 1 mM PMSF, 1 mM benzamidine] and incubated for 1-2 hours with polyclonal antibodies against NHE1 or CHP in the presence of 30 μl of protein A-Sepharose. The immune complex was washed 5 times with lysis buffer and proteins were eluted with the sample buffer containing 1% SDS. Proteins were subjected to SDS-PAGE and transferred to immobilon membranes. After incubation of membranes with antibodies, the blots were visualized by enhanced chemiluminescence (ECL) and Konica immunostaining HRP 1000. In some experiments to study the interaction between NHE1 and CHP, membranes were incubated with 100 μg of GST-CHP or MBP-NHE1 before incubation with the first antibodies.

Measurements of the Na^+/H^+ exchange activity and pH_i. The Na^+/H^+ exchange activity was evaluated by measuring EIPA-sensitive $^{22}Na^+$ uptake acitivity using stable transfectants grown to

confluence in 24-well dishes (12). For measurement of intracellular pH (pH_i)-dependence of $^{22}Na^+$ uptake, pH_i was clamped to various values by K^+/nigericin (12). Change in pH_i in response to various extracellular stimuli was monitored by equilibration of [^{14}C]benzoic acid using transfectants grown to confluence in 24-well dishes.

RESULTS (結果)

Identification of CHP-binding domain within NHE1. In order to identify the CHP-binding site within NHE1, we produced 24 MBP-NHE1 fusion proteins containing various regions of the cytoplasmic domain (aa 503-815) of human NHE1. We also produced GST-CHP fusion protein as a probe to detect the interaction between NHE1 and CHP *in vitro*. MBP-NHE1 Fusion proteins were subjected to SDS-PAGE and GST-CHP overlay assay. As a long purification step caused protein degradation, we initially checked whether CHP binds to MBP-NHE1 fusion proteins by analyzing directly total bacteria proteins without purification. We found that GST-CHP strongly bound to several MBP-NHE1 fusion proteins containing aa 503-815 (complete cytoplasmic domain), aa503-540, aa503-575 or aa 515-575 of NHE1, whereas it did not bind to other fusion proteins containing aa530-656, aa540-656 or aa560-815. These data indicated that the CHP-binding domain exists in the N-terminus of the cytoplasmic domain of NHE1. To confirm finally the CHP-binding domain, we produced three MBP-NHE1 fusion proteins containing aa 503-815 but lacking aa 515-520, 515-530 or 510-530. We found that GST-CHP did not bind to these deletion mutant proteins, although we detected a weak CHP-binding to the fusion protein lacking 515-520. Thus, we concluded that the CHP-binding domain is located in aa 515-530 of NHE1.

CHP-binding to NHE1 in vivo. In order to study the role of CHP-binding in the function of NHE1, we introduced several mutations into CHP-binding domain of NHE1. At the first, we produced three NHE1 mutants (Δ510-515, Δ515-530 or Δ510-530) deleted of aa 510-515, 515-530 or 510-530 and expressed them in the exchanger-deficient cell PS120. We immunoprecipitated the wild-type or these deletion mutant NHEs with rabbit anti-NHE1 antibody, or the endogenous CHP protein with rabbit anti-CHP antibody. We found that the wild-type NHE1 was easily detected in the immunoprecipitates with anti-CHP antibody, or vice versa, that CHP was also detected in the immunoprecipitates with anti-NHE1 antibody. As expected, NHE1 deletion mutants were not

detected in imunoprecipitates with anti-CHP antibody although a little amount of Δ510-515 was detected, indicating that aa 515-530 of NHE1 is indeed the CHP-binding domain. Unfortunately, the Na^+/H^+ exchange activity of cells expressing these deletion mutants was considerably low as compared to that of the wild-type NHE1, probably because of a severe mutation-induced change in conformation of NHE1. Therefore, we tried to introduce a more mild mutation into the CHP-binding domain of NHE1. Among more than 10 mutants we tried, a mutant protein I522C/D528C/H529C (three amino acids were substituted with cysteine) did not bind to CHP when CHP-binding ability was checked by *in vitro* GST-CHP overlay or by immunoprecipitation with anti CHP antibody. In order to determine the localization of CHP protein in the cells, we produced a plasmid for CHP conjugated with green fluorescent protein GFP, transfected it into cells and analyzed the localization of GFP fluorescence by confocal microscopy. In the exchanger-deficient cell PS120, most of GFP fluorescence was detected in the cytosol. In contrast, in PS120 cells expressing the wild-type NHE1, GFP fluorescence became to accumulate in the plasma membrane but not in the cytosol. This result suggest that NHE1 is a major target for CHP in cells. As expected, GFP fluorescence was not detected in the plasma membrane of cells expressing the mutant I522C/D528C/H529C. In addition, even in cells expressing the wild-type NHE1, GFP-fluorescence was not detected in the plasma membrane of cells co-transfected with GFP-CHPΔ160–180 that lost the binding ability to NHE1.

Characterization of NHE1 mutants lacking CHP binding

In order to study the role of CHP-binding in regulation of NHE1, we used the mutant NHE1 I522C/D528C/H529C that lost the binding to NHE1, but retained a relatively high activity of the Na^+/H^+ exchange. The pH_i-dependence of EIPA-sensitive $^{22}Na^+$ uptake of cells expressing I522C/D528C/H529C was almost the same as that of cells expressing the wild-type NHE1. Depletion of cell ATP strongly inhibited $^{22}Na^+$ uptake activity by inducing an acidic shift of pH_i dependence of this mutant, like the wild-type NHE1. In cells expressing NHE1, various extracellular stimuli are known to induce a long-lasting cytoplasmic alkalinization through activation of NHE1. We found that the cytoplasmic alkalinization was also observed in cells expressing I522C/D528C/H529C in response to all the stimuli tested (thrombin, PDGF, PMA, hyperosmolarity and LPA), suggesting that CHP-binding is not involved in the regulation of NHE1. Next we tested the localization of this mutant by confocal microscopy. Most of the wild-type NHE1 protein was localized in the plasma

membrane. In contrast, a relatively high amount of I522C/D528C/H529C protein was detected in the endomembranous structure around the nucleus as well as the plasma membrane. Our preliminary study revealed that this endomembranous structure is the Golgi membranes. Consistent with this finding, we found that more than 50% of the I522C/D528C/H529C protein expressed in cells existed as an immature form with the molecular weight of 90 kD. The data suggest that CHP-binding may be involved in protein processing of NHE1.

DISCUSSION (考察)

In this study, we identified the CHP-binding domain within NHE1. The identified CHP-binding domain (aa 515-530) is located in the N-terminus of the cytoplasmic domain of NHE1. The cytoplasmic domain of NHE1 has previously been shown to be separated into at least four functional domains (aa 515-595, aa 595-635, aa 635-656, aa 656-815). Subdomain I (aa 515-595) has been shown to play an important role in pH_i-maintenance, ATP-depletion-induced inhibition of NHE1 and growth factor-induced activation of NHE1. It is likely that distinct smaller regions within subdomain I are involved in these multiple physiological roles. The present data indicates that a part of subdomain I is also involved in the interaction with CHP.

In this study, we expressed a mutant exchanger I522C/D528C/H529C that has no CHP-binding ability but has a relatively high Na^+/H^+ exchange activity. However, this mutant was similar to the wild-type NHE1 in following points; 1) pH_i dependence of exchange, 2) ATP depletion-induced of inhibition of exchange and 3) cytoplasmic alkalinization in response to various stimuli. These findings suggest that CHP-binding is not required for such functions of NHE1. Our data are largely different from the previous report (10). According to the previous report (10), CHP is an inhibitory protein and released from NHE1 through dephosphorylation of CHP upon stimulation of cells. However, we did not observed an alkaline shift in pH_i dependence of exchange in cells expressing I522C/D528C/H529C, that is expected from CHP-induced inhibition of exchange. We also observed a strong acidic shift of pH_i dependence induced by ATP depletion and cytoplasmic alkalinization in response to thrombin, PDGF, hyperosmolarity (200 mM sucrose) or PMA. These results are consistent with our previous reports that the former phenomenon is attributable to a limited fragment

of aa 540-560 (Ikeda et al. unpublished observation), whereas the latter one is attributable to aa 566-635 (13).

At present, the precise role of CHP is not known. However, the finding that a part of the I522C/D528C/H529C protein is concentrated in Golgi-like structure in cells, suggest that CHP may be involved in protein processing. It is possible that the CHP-binding accelerates the translocation of NHE1 from Golgi cisternae to the plasma membrane, although further study would be required. This concept was also suggested from a recent report that p22 (the same protein as CHP) is involved in vesicle traffic (14). Another possible explanation for the role of CHP came from confocal microscopy experiment with GFP-CHP. We observed that GFP-CHP accumulated in the plasma membrane only if we expressed NHE1 in PS120 cells, indicating that NHE1 is a major target for CHP. This finding leads to a novel concept that NHE1 is required as a scaffolding protein for recruitment of CHP to the plasma membrane. Future experiments would determine the precise role of CHP-binding to to NHE1.

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2D-W03-06　Na⁺/H⁺ exchanger(NHE1)におけるカルシニューリン類似タンパク質の結合部位とその役割　Tianxiang Pang、○若林繁夫、重川宗一（国立循環器病センター研）

細胞形質膜に普遍的に発現する Na⁺/H⁺ exchanger (NHE1)は、増殖因子や高浸透圧等、様々な刺激によって活性化されることが知られる。活性化の全貌はまだ明らかでないが、NHE1 の細胞質ドメインとカルモデュリンやその他の制御因子との相互作用が重要と考えられている。NHE1 に結合するタンパク質としてカルシニューリン類似タンパク質(CHP)が最近クローニングされたが(PNAS、93:12631,1996)、その NHE1 における正確な結合部位と活性調節における役割には疑問が残された。我々は、CHP 抗体、GST-CHP 融合タンパク質、NHE1 の様々な MBP 融合タンパク質を作成し、in vitro オーバーレイアッセイ、共免疫沈降法によって、NHE1 と CHP との相互作用を検討した。その結果、NHE1 細胞質ドメイン中の膜貫通ドメインに近いアミノ酸 515-530 が CHP 結合部位として同定された。この領域は、以前報告されたもの(aa566-635)とは異なる。我々は最終的に、細胞に発現した変異タンパク質Δ515-530(aa515-530 を欠失)と CHP との結合が検出できない程弱いことを確認した。また免疫染色の結果、この変異タンパク質Δ515-530 の大部分は細胞内膜系(恐らく Golgi)にとどまっており、NHE1 の膜へのターゲティングにおける CHP の関与が想定される。しかし一方で、この領域は我々が以前報告した pHi感受性維持、ATP 感受性、増殖因子活性化等に関与するサブドメイン I(aa503-595)の中にあり、CHP が NHE1 の活性調節に関与する可能性もあり、現在より詳しい解析を行っている。