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学位（専攻分野）	博士（理学）
学 位 記 番 号	総研大甲第975号
学位授与の日付	平成１８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	Nitric-oxide-induced activation of TRPC5 Ca^{2+} channel mediates a positive feedback loop in receptor-induced eNOS signaling
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論文内容の要旨

Nitric-oxide-induced activation of TRPC5 Ca^{2+} channel mediates a positive feedback loop in receptor-induced eNOS signaling

[INTRODUCTION]

Ca^{2+} and nitric oxide (NO) form key signals that are precisely coordinated to control a wide variety of biolo gical responses. Here, He describes a novel mechanism underlying the crosstalk between the Ca^{2+} and NO signals. Mammalian members of the classical transient receptor potential channel type 5 (TRPC5), a member of TRPC family that have been categorized as Ca^{2+}-permeable cation channels receptor-activated via phospholipase C (PLC), mediated Ca^{2+} entry in response to NO-releasing agents. TRPC5 was also responsive to reactive oxygen species (ROS) and cysteine-selective oxidants.

NO production by NO synthases (NOS) is under complex and tight control. NOS isoforms have been distinguished from the basis of their Ca^{2+}-dependence (eNOS and nNOS) or -independence (iNOS): elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers NO production by Ca^{2+}-dependent NOSs. But it is still controversial whether the calmodulin (CaM)-mediated activation of NOS requires specific modes of upstream Ca^{2+} signaling through particular subtypes of Ca^{2+} release channels and/or receptor-activated Ca^{2+}-permeable cation channels (RACCs)/TRPC Ca^{2+}-permeable channels. Furthermore, mode of feedback regulation of Ca^{2+} signaling by NO remains unclear: both positive and negative regulation by NO of Ca^{2+} mobilizing pathways including RACCs have been displayed. Thus, to address these issues for understanding physiological regulation of NO production by Ca^{2+} signaling, it is crucial that Ca^{2+} influx pathways selectively linked to NOS activation/NO production upon receptor activation are identified.

[RESULTS]

He has examined activity of TRPC5 channel recombinantly expressed in HEK cells using patch-clamp technique and the fluorescent Ca^{2+} indicator fura-2 for measurements of intracellular free calcium concentration ($[Ca^{2+}]_i$). Strikingly, HEK cells expressing TRPC5 were responsive to NO donors such as S-nitroso-N-acetyl-DL-pnocillamine (SNAP) at concentrations >10 μM or 5-Nitro-2-PDS, which selectively recognizes cysteine residues (free sulfhydryl groups). Application of reducing agent DTT suppressed the 5-nitro-2-PDS-induced Ca^{2+} response after a time lag that decreased with increment of DTT concentration. They have previously demonstrated activation of TRPC5 channels is triggered upon stimulation of endogenous ATP receptor P2Y linked to Gq/PLC β and Ca^{2+} release from ER via IP_3 receptors. Therefore, they examined a possibility that PDS targets one of the regulatory molecules of the signaling cascade in triggering TRPC5 activation, by employing their blockers and dominant negative suppressors.

TRPC5-mediated $[Ca^{2+}]_i$ increase evoked by 5-nitro-2-PDS remained intact in cells pretreated with the PLCβ blocker U73122. Furthermore, TRPC5 elicited similar 5-nitro-2-PDS-evoked $[Ca^{2+}]_i$ responses in wild-type chicken DT40 lymphocytes and in DT40 lymphocytes deficient of all the three IP_3 receptor subtypes. They examined another possibility that PDS directly acts on TRPC5 channel complex. Cell-excised patch clamp recording and labeling experiments using TRC5 mutants indicated that direct modification of the site Cys553 accessible from cytoplasmic side is responsible for activation by NO and cysteine-selective oxidants in the TRPC5 protein.

In bovine aortic endothelial cells treated with all-*trans*-retinoic acid (RA) or recombinantly expressing TRPC5, ATP receptor stimulation caused significant production of nitric oxide (NO) derived from endothelial nitric oxide synthase (eN OS) and further $[Ca^{2+}]_i$ rises. RNA interference targeting bovine TRPC5 attenuated ATP-induced $[Ca^{2+}]_i$ rises and NO production in cultured endothelial cells treated with RA. Hence, TRPC5 may play an essential role in mediating Ca^{2+} entry that selectively elicits NO production in response to receptor stimulation in endothelial cells. He studied feedback effects of NO upon Ca^{2+} signaling in cultured endothelial cells, since NO activates TRPC5 Ca^{2+} channels in the recombinant system as described above. NOS inhibitor L-NAME and NO quencher carboxy-PTIO markedly suppressed ATP-induced Ca^{2+} entry. Interestingly, TRPC5 and eNOS significantly affected each other's pattern of subcellular distribution to colocalize in HEK cells. eNOS-GFP was concentrated at the plasma membrane by TRPC5 coexpression, while eNOS-DsRed was more evenly distributed through the cytoplasmic area when expressed alone. Localization of TRPC5-GFP at the plasma membrane was punctuated in eNOS-coexpressing cells, while it showed continuous plasma membrane distribution without eNOS coexpression. The results suggest that TRPC5 and eNOS form a protein complex, which controls feedback regulation of TRPC5 by NO. Immunocytochemical studies indicate colocalization of native TRPC5 and eNOS at near plasmamembrane in cultured endothelial cells. Caveolin-1 showed a localization overlapping with TRPC5, consistent with the previously demonstrated complexation of eNOS with caveolin-1. In cultured endothelial cells, the recombinant construct TRPC5-GFP was co-immunoprecipitated with the eNOS-FLAG construct, while native caveolin-1 was co-immunoprecipitated with the TRPC5-GFP construct. Furthermore, immunohistochemical studies performed for the whole mount preparation of mesenteric artery revealed colocalization of native TRPC5 and eNOS in endothelial cell layer. Thus, the TRPC5-eNOS complex can be formed at the caveolin-1-rich plasmamembrane area of endothelial cells.

[DISCUSSION]

The present study describes NO-induced activation of TRPC5, which has been categorized as Ca^{2+} channels activated upon stimulation of PLC-coupled receptors. The cysteine residue 553 in TRPC5 has been revealed as the action site of NO by using

cysteine-specific oxidants PDSs that share common binding site with NO. In endothelial cells, TRPC5 play an essential role in receptor-induced NO production by mediating Ca^{2+} entry necessary for Ca^{2+}-dependent activation of eNOS. They propose that NO-induced TRPC5 activation mediates a positive feedback loop in the eNOS activation cascade that controls NO production. A protein complex formation of TRPC5 and eNOS revealed by co-immunoprecipitation experiments may be an important molecular basis that underlies this physiological significance of TRPC5.

[CONCLUSIONS]

These results suggest that TRPC5 is activated by receptor stimulation via eNOS, leading to an idea that NO generated by nearby eNOS activates TRPC5 in caveolae. There is a positive feedback cycle between TRPC5 and eNOS that amplifies NO production and $[Ca^{2+}]_i$ rises through TRPC5 nitrosylation.

論文の審査結果の要旨

TRP (transient receptor potential) チャネルは、さまざまな細胞応答現象に関与する重要なイオンチャネルであり、活性化機構が多岐にわたることが特徴である。申請者吉田卓史による本論文は、TRPC5 チャネルを対象とし、Ca^{2+}、および NO（一酸化窒素）シグナリングの調節における役割を解析したものである。

TRPC5 は、phospholipase C によって活性化されることが既に知られているが、本論文において申請者は、TRPC5 が、NO によってもニトロシル化を介して活性化されることを明らかにした。また、TRPC5 チャネルを通って細胞内に流入した Ca^{2+} が NO 合成酵素である eNOS を活性化することも見いだし、これらふたつの知見に基づいて、ポジティブフィードバック機構が存在することを結論した。さらに、TRPC5 と eNOS および caveolin-1 が細胞膜上で共局在し、また免疫共沈することから、TRPC5 と eNOS が複合体を形成していること、そしてその細胞膜上の場が、caveolae 領域であるということを結論した。これらの成果は、細胞生理学的に大きな意義を持ち、さらなる研究への礎となるものである。

申請者は、また、構造機能連関の側面からもアプローチし、点変異体を用いた解析の結果に基づいて、TRPC5 チャネルのニトロシル化の一次構造上の基盤が、第 5 膜貫通領域とチャネルポア領域の間に位置する C553 と C558 であることを明らかにした。C553 と C558 は、他のイオンチャネルから類推すると細胞内に面しているとは考えられない位置にあったため、これらの残基が、細胞内側からのみ NO 供与体等の Cys 残基修飾剤によって修飾されるという知見は極めて興味深く、TRP チャネルのポアおよび周辺領域の特異な構造を示唆するものである。

以上のように、本論文は、分子生物学、生化学、細胞生物学、生理学等多岐にわたる、十分な学際的実験結果に基づき、TRPC5 を介する Ca^{2+} と NO のシグナリングのクロストーク、ポジティブフィードバックの機構、さらに NO 修飾の構造基盤を明らかにしたものであり、明確な新奇性を持った、優れた論文である。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。