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論文内容の要旨

In B lymphocytes, the engagement of B cell receptors triggers elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) which controls cell proliferation, differentiation and apoptotic processes. The ligation of BCR activates phospholipase C (PLC) γ2. PLCγ2 hydrolyses phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces rapid Ca^{2+} release from the intracellular store of endoplasmic reticulum (ER) and depletion of the store of Ca^{2+} contents, which subsequently activates "store-operated" Ca^{2+} channels (SOCs) or Ca^{2+} release activated Ca^{2+} (CRAC) channels. In T lymphocytes, electrophysiological analyses strongly suggest that CRAC channels are solely responsible for Ca^{2+} influx during T cell activation. Furthermore, in patients with severe immunodeficiency, non-responsive T cells display a specific defect in Ca^{2+} influx associated with the absence of I_{CRAC}. I_{CRAC} current has also been reported in the avian B cell line DT40. In addition, previous work also demonstrated that CRAC channels are required for BCR-mediated Ca^{2+} signaling such as BCR-induced Ca^{2+} oscillations and NF-AT activation. By analogy to TCR-induced Ca^{2+} influx, CRAC channels are thought to be the sole Ca^{2+} influx pathway in B lymphocytes. However, B cells isolated from the above-mentioned patients with a defect in CRAC activity appear to be capable of mounting normal immune responses. These results suggest the existence of an alternative BCR-induced Ca^{2+} influx pathway. A candidate for this Ca^{2+} influx pathway is DAG-activated Ca^{2+} entry. While DAG is classically known to be the activator of protein kinase C, Hofmann *et al.* reported that DAG directly activates plasma membrane resident Ca^{2+} channels. The molecular entities of SOCs or DAG-activated Ca^{2+} channels are canonical transient receptor potential (TRPC) cation channels, which is the mammalian homolog of the *Drosophila trp* gene. Among seven TRPC family members, TRPC2, TRPC3, TRPC6 and TRPC7 have been reported to be activated by DAG. For the physiological importance of these DAG-activated channels, DAG-activated TRPC channels function as nonselective cation channels to induce membrane depolarization, which in turn activates voltage-dependent Ca^{2+} channels. Therefore, the physiological importance of Ca^{2+} ion entering through the DAG-activated TRPC channels is largely unknown. To investigate the physiological significances of DAG-activated Ca^{2+} channels, he analyzed the effect of DAG-activated TRPC3 knockout on BCR signaling in avian DT40 B cells.

Firstly, he compared BCR-induced $[Ca^{2+}]_i$ changes in single WT and TRPC3-deficient DT40 B cells using digital video fluorescence imaging of the Ca^{2+}-sensitive dye fura-2. In the presence of 2 mM extracellular Ca^{2+}, BCR-induced $[Ca^{2+}]_i$ increase was significantly reduced in TRPC3-deficient cells. In contrast, there is no difference in the averaged BCR-induced $[Ca^{2+}]_i$ rises between WT and TRPC3-deficient cells in the absence of extracellular Ca^{2+}. Therefore, deficient

BCR-induced Ca^{2+} mobilization in TRPC3-deficient cells is causative to the defect of Ca^{2+} influx activated by BCR stimulation. He next examined DAG-activated Ca^{2+} entry using the membrane permeable DAG analog, 1,2-oreoyl-sn acetyl glycerol (OAG). OAG-induced Ca^{2+} response was attenuated in TRPC3-deficient cells compared to WT cells. To make a comparison of OAG-induced channel activity between WT and TRPC3-deficient cells, he analyzed OAG-induced cation current using a whole-cell patch-clamp configuration. As a result, the current density of OAG-induced cation currents was significantly lowered in TRPC3-deficient cells compared to WT. Furthermore, he analyzed whether the defect of BCR-induced Ca^{2+} entry in TRPC3-deficient cells is also attributable to the defect of SOCE using ionomycin (IM), Ca^{2+} ionophore that fully depletes intracellular Ca^{2+} stores and elicits SOCE. However, the loss of TRPC3 does not affect SOCE. To confirm the effect of TRPC3 knockout on SOCE, Ca^{2+} release activated current (I_{CRAC}) were analyzed using whole-cell patch-clamp techniques. Consistent with the result obtained by Ca^{2+} imaging, peak current density and half maximal activation time of I_{CRAC} recorded in TRPC3-deficient cells were comparable to those in WT cells. These results clearly indicate that endogenous TRPC3 forms DAG-activated Ca^{2+} permeable cation channels in DT40 B lymphocytes, but is not involved in SOC channels.

Previous study revealed that Ca^{2+} oscillations required Ca^{2+} influx-dependent secondary activation of PLCγ2 and secondary production of IP_3 accompanied with the membrane translocation of PLCγ2. TRPC3 is a candidate of molecular entity of that Ca^{2+} influx, based on the observation that PLCγ2 functionally and physically coupled to TRPC3 in heterologous expression system. He next evaluated whether native TRPC3 can also functionally associate with PLCγ2 in native DT40 cells using PLCγ2-EYFP and time-lapse confocal laser microscopy. The ligation of BCR by the anti-IgM induced membrane translocation of PLCγ2-EYFP in WT cells. In contrast, TRPC3-deficient cells exhibited nearly abolished PLCγ2-EYFP membrane translocation. Consistent with this suppressed BCR-induced PLCγ2 translocation in TRPC3-deficient cells, the amplitudes of Ca^{2+} oscillations were reduced compared to WT DT40 lymphocytes. Furthermore, TRPC3-deficeint cells showed about 30% reduction of BCR-induced NF-AT activation compared to WT DT40 cells. These results clearly indicate the critical role of DAG-activated TRPC3 channels in BCR-induced Ca^{2+} signaling.

The ligation of BCR with anti-IgM induces extracellular-regulated kinase (ERK), a mitogen-activated protein kinase, phosphorylation in a Ca^{2+} influx and DAG-dependent manner. Therefore, he examined whether TRPC3 knock out affects BCR-induced ERK activation using phosphospecific ERK antibody. TRPC3-deficient cells exhibited suppressed ERK activation, and this defect was resolved by the heterologous expression of mouse TRPC3. These results indicate that Ca^{2+} influx via DAG-activated TRPC3 channels is required for full activation of ERK in DT40 B

lymphocytes. To reveal the target molecule of Ca^{2+} influx via TRPC3 channels for BCR-mediated ERK activation. He focused on protein kinase Cβ, and analyzed membrane translocation of PKCβ, which is the most critical step in the activation mechanism of PKC and can be used for measuring PKC activation, using the membrane fractionation method and confocal visualization of PKCβ-EGFP. In these experiments, while WT cells showed sustained BCR-induced membrane translocation of PKCβ, TRPC3 cells exhibited transient membrane translocation. Therefore TRPC3-mediated Ca^{2+} entry is required for sustained PKCβ membrane translocation.

In conclusion, he reports here that TRPC3 forms DAG-activate Ca^{2+} channels in DT40 B lymphocytes and TRPC3-mediated Ca^{2+} influx regulate "sustained" BCR-induced Ca^{2+} and DAG signaling via regulating the "sustained" membrane translocation of key signaling molecules, PLC γ2 and PKCβ respectively. To date, it has been accepted that BCR-induced Ca^{2+} influx is solely responsible for SOC channels. However, the Ca2+ influx mechanisms are more complex than previous thought. He clearly showed that DAG-activated TRPC3-mediated Ca^{2+} influx is also activated by BCR stimulation and regulates the especially "sustained" phase of both Ca^{2+} and DAG signaling in DT40 cells. NF-AT, the read out of Ca^{2+} signaling, is required for the T and B cell development and programmed cell death. Also, "sustained" or transient ERK activation, the read out of DAG signaling, is required for the determining the cell proliferation or differentiation in many cells Therefore, TRPC3 plays a crucial role in B cell development in which cell proliferation, differentiation an d even cell death are repeated all the time.

論文の審査結果の要旨

B 細胞受容体（BCR）刺激は、免疫 B 細胞において、ホスホリパーゼ C (PLC)γ2 の活性化を引き起こし、細胞外からの Ca^{2+} 流入を惹起する。しかし、これまで考えられていた以外にも、Ca^{2+} 流入経路の存在が示唆されてきたが、チャネルを介した Ca^{2+} 流入の生理的意義およびその分子実体は明らかにされていない。本研究では TRPC3 欠損ニワトリ免疫 B 細胞株 DT40 を作製し、wild type 細胞と比較することによって、B 細胞受容体 (BCR)シグナル伝達系における内在性 DAG 活性化 Ca^{2+} 流入を突き止め、その生理的意義を明らかすることを目的としている。具体的成果の内容は以下に示す。

BCR 刺激は、DT40 細胞において一過的な Ca^{2+} 上昇と、持続的な Ca^{2+} オシレーションを誘導した。BCR 刺激に惹起される Ca^{2+} オシレーションの発生に必要な "Ca^{2+} 流入依存的な PLCγ2 の形質膜への集積" が TRPC3 ノックアウト細胞において抑制され、Ca^{2+} オシレーション、および Ca^{2+} 依存的転写因子である NF-AT の活性化が抑制されていた。さらに TRPC3 の欠損により、BCR に誘導される extracelllular signal-regulated kinase (ERK) の持続的な活性化が抑制されることを明らかにしている。PKCβの持続的な膜移行は、TRPC3 の欠損により抑制されていたことから、ERK の抑制は PKCβの持続的な膜移行および活性化の抑制によるものであることが示唆された。以上のことから、TRPC3 は PKCβの BCR 刺激後の持続的な形質膜への局在に必要となる Ca^{2+} 供給を担うと共に、PKCβの形質膜へのアンカーとして機能することを示している。さらに、BCR 刺激に誘導される MAPK 経路の活性化には、ストアからの放出および store operated Ca^{2+} 流入は関与していないことを示唆した結果を得ている。つまり、DAG に活性化される TRPC3 を介した Ca^{2+} 流入は、BCR に誘導される Ca^{2+} および MAPK 経路において、主要な Ca^{2+} 依存的シグナル分子の膜移行を制御し、その持続的な活性化に重要な役割を果たしていると考えられる。これらの結果から、B 細胞の発達過程において繰り返される細胞の増殖、分化あるいは細胞死において、TRPC3 は重要な役割を果たしていることが推測される。

研究内容は、免疫系 B 細胞における TRPC3Ca^{2+} チャネルの存在および生理学的重要性を明確に示したものであり、この分野の研究に明確な方向性を示している。また、研究目的も明確で実験手法および研究の進め方も適切である。論旨も理論的で内容も学問的に高いものであり、博士論文として十分に値するものである