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Ca^{2+} oscillation induced by P2Y2 receptor activation and its regulation by a neuron-specific subtype of PKC (γPKC)

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ABSTRACT

We found that stimulation of P2Y2 receptor (P2Y2R), which is endogenously expressed in CHO-K1 cells, induced intracellular calcium ($[Ca^{2+}]_i$) oscillation with a low frequency of 11.4 ± 2.7 mHz. When CHO-K1 cells expressing GFP-tagged kinase-negative γPKC (γPKC-KN-GFP), which is a neuron-specific subtype of PKC, were stimulated with UDP, γPKC-KN-GFP, but not wild-type γPKC (γPKC-GFP) showed an oscillatory translocation. The oscillatory translocation of γPKC-KN-GFP corresponded with $[Ca^{2+}]_i$ oscillation, which was not observed in the cells expressing γPKC-GFP. We examined the mechanism of P2Y2R-induced $[Ca^{2+}]_i$ oscillation pharmacologically. γPKC-KN-GFP oscillation was stopped by an extracellular Ca^{2+} chelator, EGTA, an antagonist of P2Y2R, Suramin, and store-operated calcium channel (SOC) inhibitors, SKF96365 and 2-ABP. Taken together, P2Y2R-induced $[Ca^{2+}]_i$ oscillation in CHO-K1 cells is related with Ca^{2+} influx through SOC, whose function may be negatively regulated by γPKC. This $[Ca^{2+}]_i$ oscillation was distinct from that induced by metabotropic glutamate receptor 5 (mGluR5) stimulation in the frequency (72.3 ± 5.3 mHz) and in the regulatory mechanism.

Protein kinase C (PKC) family comprises 10 subtypes. The conventional subtypes (cPKC; α, βI, βII, γ) are Ca^{2+} and DAG dependent, in contrast, novel subtypes (nPKC: δ, ε, η, θ,) are DAG dependent, but Ca^{2+} independent. PKC plays pivotal roles in many signaling pathways, and the existence of multiple subtypes suggests the subtype-specific function. γPKC is restrictedly expressed in brain and spinal cord and believed as a neuron-specific subtype. Recently, we reported that activation of metabotropic glutamate receptor 5 (mGluR5) elicits oscillatory $[Ca^{2+}]_i$ increase, oscillatory γPKC-GFP translocation between cytoplasm and plasma membrane, and transient, but not oscillatory, δPKC-GFP translocation from cytoplasm to the plasma membrane [23]. This $[Ca^{2+}]_i$ oscillation and oscillatory translocation of γPKC-GFP are blocked by the activation of δPKC but not by γPKC [23]. The report is a good example showing that $[Ca^{2+}]_i$ oscillation is regulated by orchestration of subtype-specific function of PKC. Recently, importance of $[Ca^{2+}]_i$ oscillation resulting in physiological functions has been reported in many cell-types, such as glial cells [10], endothelial cells [14], and cardiomyocytes [2].

P2Y purinoceptors (P2YRs), which are activated by extracellular nucleotides, belong to the superfamily of G-protein-coupled receptors (GPCRs). The P2YR family is composed out of eight mammalian subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) and subdivided into the five $G_{(q)}$-coupled subtypes (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11), resulting in generation of IP3 and DAG, and three $G_{(i)}$-coupled subtypes (P2Y12, P2Y13, and P2Y14) [5,27]. P2Y11 receptors can additionally couple to $G_{(s)}$ [3,5]. P2Y2R has a wide tissue distribution [27] including brain (NCBI, UniGene, Gene Expression Profile) and its function is reported in many cell-types, such as glial cells [7], airway epithelial cells [5], and coronary artery smooth muscle cells [19].

In the first stage of the present study, we found that stimulation of P2Y2R, which is endogenously expressed in CHO-K1 cells, induced a low-frequent oscillatory translocation of γPKC-KN-GFP and intracellular calcium $[Ca^{2+}]_i$ oscillation (11.4 ± 2.7 mHz). This $[Ca^{2+}]_i$ oscillation was distinct from mGluR5-induced $[Ca^{2+}]_i$ oscillation in the frequency (72.3 ± 5.3 mHz), which we previously reported [23]. For understanding of the physiological importance of distinct types of $[Ca^{2+}]_i$ oscillations through distinct receptors, we examined the mechanism of P2Y2R-induced $[Ca^{2+}]_i$ oscillation. P2Y2R-mediated $[Ca^{2+}]_i$ oscillation depended on Ca^{2+} influx from extracellular space, and that this Ca^{2+} influx was regulated by kinase-activity of γPKC. Moreover, our experiment using various

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kinds of Ca^{2+} channel inhibitors suggests that the $[Ca^{2+}]_i$ oscillation may be regulated by a store-operated calcium channel (SOC). Taken together, P2Y2R-induced $[Ca^{2+}]_i$ oscillation is related with Ca^{2+} influx through SOC, whose function may be regulated by γPKC.

Uridine-5′-triphosphate sodium (UTP), uridine-5′-diphosphate sodium (UDP), and EGTA were purchased from Nacalai Tesque. 2-Amino-ethoxydiphenylborate (2-ABP) was from ALEXTS Biochemical. Go6983, thapsigargin, and SKF96365 were from Calbiochem. Verapamil and Fura-2AM were from Dojindo. Dantroren and Suramin were from Sigma–Aldrich.

C-terminally GFP-tagged γPKC and DsRed-tagged γPKC, designated γPKC-GFP and γPKC-DsRed, were previously described [18,23]. cDNA fragment encoding kinase-negative mutant of γPKC (γPKC-KN), which has a mutation at ATP binding site (K380M) to avoid phosphorylation of substrate, was produced by PCR-mediated site-directed mutagenesis. This fragment was cloned into the GFP vector instead of γPKC, and the C-terminally GFP-tagged γPKC-KN was designated γPKC-KN-GFP. The construct was sequenced to confirm its identity, and assayed to confirm its activity as a kinase-negative mutant (data not shown). N-terminal fragment of δPKC (nt: 1–1001) adapted with EcoRI/BglII sites was obtained from δPKC-GFP, which is described previously [26], and C-terminal fragment of δPKC (nt: 1002–2022) adapted with BglII/SalI sites was produced by PCR. An orange fluorescent protein, monomeric Kurabira Orange (mKO) [24] adapted with BstXI/XbaI were produced by PCR. The two fragments of δPKC were cloned into EcoRI/SalI site of MCS of pIRES2-DsRed2 (Clontech), and the mKO fragment was cloned into pIRES2-DsRed2 in place of DsRed2, and designated δPKC-IRES-mKO.

CHO-K1 cells from Health Science Research Resources Bank (Osaka, Japan) were maintained in Ham's F-12 medium (Nacalai Tesque) supplemented with 10% FBS (Invitrogen) and antibiotics (100 U/ml penicillin and streptomycin), at 37 °C in 5% CO_2. CHO-K1 cells were seeded at a density of 1×10^5 cells on 35-mm glass-bottomed culture dishes (MatTek), incubated for 24 h before transfection, and then transfected with indicated plasmid using FuGENE6 (Roche).

CHO-K1 cells transfected with GFP-tagged PKC construct were cultured for 24 h. The media was replaced with HEPES buffer [26]. Translocation of γPKC-GFP-KN or γPKC-GFP was triggered by the addition of the stimulant to the HEPES buffer to obtain the appropriate final concentration. To pre-treat cells with inhibitor, the culture medium was replaced by HEPES buffer containing indicated concentration of inhibitor and pre-incubated for the indicated time. For application of inhibitor during imaging study, HEPES buffer containing inhibitor was added to each dish to obtain the indicated final concentration. All experiments were performed using a LSM 510 invert confocal laser scanning fluorescence microscopy (Carl Zeiss) at 37 °C with a heated stage and objective [26]. Time series images were recorded before and after stimulation for 10 min.

Changes in the levels of $[Ca^{2+}]_i$ were measured using Fura-2AM and Argus/HiSCA (Hamamastu Photonics) with heated objective [26]. Briefly, transfected CHO-K1 cells were grown on 35-mm glass-bottomed culture dishes. Cells were incubated with 5 μM of Fura 2-AM at 37 °C for 1 h. After washing cells, sequential images were captured at wavelengths above 510 nm after excitation at 340 and 380 nm for $[Ca^{2+}]_i$ monitoring. The signal was expressed as the 340:380 ratio of Fura-2 fluorescence.

To examine the endogenously expressed PKC isoforms in CHO-K1 cells, cells grown in 100-mm dish were lysed in ice-cold homogenizing buffer containing protease inhibitors by sonication [25]. After centrifugation at $2000 \times g$ for 10 min at 4 °C, the supernatants were subjected to SDS-PAGE and followed by immunoblotting using anti-αPKC (C20), anti-γPKC (C19), anti-εPKC (C15), anti-μPKC (C20), anti-ηPKC (C15) (1/500, Santa Cruz Biotechnology), anti-βPKC (1/500; [8]), anti-δPKC (1/500; Transduction Laboratories), or anti-ζPKC (1/500; UPSTATE) for 2 h at room temperature. The immunoreactive bands were visualized with peroxidase-conjugated secondary Ab (1/10,000) by an ECL detection kit (GE Healthcare).

RT-PCR was used to examine the endogenously expressed subtype of P2YR using SuperScript III RT-PCR system (Invitrogen). Total RNA was isolated from CHO-K1 cells using Trizol (Invitrogen). One microgram of total RNA was subjected to each RT reaction. For the PCR amplification, 2 μl of the cDNA products of the RT reaction was denatured for 5 min at 95 °C prior to 40 cycles at 95 °C for 1 min, 63 °C for 1 min, 72 °C for 1 min. The primer sets used for PCR were 5′-CCTAGCTCCTCAGTTTGTAACATGG-3′ and 5′-GTACCACAGTTGGTGGAGATTGAG-3′ (expected band size: 516 bp), and 5′-ATGTTCAATTTGGCTCTGGC-3′ and 5′-CTGTTGAGACTTGCTAGACCTCTTGT-3′ [13] (expected band size: 665 bp) for P2Y1R, 5′-GCAGGAGCTGATCGGGTCCAG-3′ and 5′-CTGGTGGTGACAAAGTAGAGCAC-3′ (expected band size: 538 bp) for P2Y2R, 5′-CTCTGTCTGATGCCATCAGTCCC-3′ and 5′-GCACGAGGCAGCCAGCTACTAC-3′ (expected band size: 535 bp) for P2Y4R, 5′-GACATCTTCCATCTTGCATGAGACAG-3′ and 5′-AGGCACTGGGCTGTCACAGC-3′ (expected band size: 538 bp) for P2Y6R, and 5′-ATGGCCGCCACCACTTCAG-3′ and 5′-CCTTTTCAGGTGCGAGAAGCTGAG-3′ (expected band size: 493 bp) for P2Y11R. These primer sets were designated using highly conserved regions in human, mouse, and rat P2YR cDNA-sequences deposited in GeneBank. The identities of these PCR products were confirmed by sequencing.

When P2YR in CHO-K1 cells expressing γPKC-GFP was stimulated with 100 μM UTP, γPKC-GFP translocated to the plasma membrane, returned to the cytoplasm in 60–120 s, and stayed there at least 600 s after the stimulation (Fig. 1A, left), as reported previously [20]. Interestingly, UTP-stimulation of CHO-K1 cells expressing γPKC-KN-GFP induced the oscillatory translocation of γPKC-KN-GFP between cytoplasm and the plasma membrane after the first and strong translocation to the plasma membrane (Fig. 1A, right). The results suggested that the oscillation of γPKC-KN-GFP was induced by the inhibition of the kinase-activity of γPKC. The frequency of the oscillatory translocation was 11.4 ± 2.7 mHz and the amplitude of the translocation was significantly lower than the first translocation (about 30% of the first translocation). Then we examined the effect of PKC inhibitor on the translocation of γPKC-GFP when stimulated with 100 μM UTP. Pre-treatment for 10 min with 500 μM Go6983, a cPKC inhibitor, induced the oscillatory translocation of γPKC-GFP (Fig. 1B). Similar results were obtained using 100 μM UDP (Fig. 1C) and 100 μM ATP (data not shown) instead of UTP.

It is known that γPKC translocates to the plasma membrane in response to phospholipids breakdown by phospholipase C. Although members of nPKC, Ca^{2+}-independent PKC subtypes, show membrane translocation without $[Ca^{2+}]_i$ increase, receptor-mediated translocation of γPKC requires $[Ca^{2+}]_i$ rise [18]. To examine whether the translocation of γPKC-KN-GFP is accompanied by Ca^{2+} oscillation or not, we measured $[Ca^{2+}]_i$ change after the stimulation of P2YR with 100 μM UDP in CHO-K1 cells expressing γPKC-GFP, γPKC(KN)-GFP or GFP alone. Cells expressing γPKC-KN-GFP (11.0 ± 0.7 mHz) or GFP (10.2 ± 0.9 mHz) showed oscillatory change of $[Ca^{2+}]_i$, however, cells expressing γPKC-GFP did not show the $[Ca^{2+}]_i$ oscillation (Fig. 1D). These results suggest that the oscillatory translocation of γPKC-KN-GFP after stimulation of P2YR is associated with kinase-activity of γPKC: kinase-activity of γPKC blocks the oscillatory $[Ca^{2+}]_i$ increase and oscillatory γPKC translocation.

Extracellular nucleotides, UDP, UTP, and ATP induced transient translocation of γPKC to the plasma membrane, suggesting that