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ORIGINAL ARTICLE

ROLE OF PROTEIN KINASE C IN ACETYLCHOLINE-INDUCED CA^{2+} INFLUX UNDER ENHANCED PHOSPHOLIPASE C-δ1

Kazuo Murakami, Tomohiro Osanai, Makoto Tanaka, Takahiko Kinjo, Tomohiro Tanno, Ikuyo Narita, Misato Kawai, Hirofumi Tomita, and Ken Okumura

Abstract ***Background:*** We reported that enhanced phospholipase C (PLC)-δ1, which was detected in patients with coronary spasm, caused coronary spasm in mice. We investigated the role of protein kinase C (PKC) in acetylcholine (ACh)-induced Ca^{2+} influx under enhanced PLC-δ1 using human embryonic kidney (HEK)-293 cells.
Methods and Results: Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by fura-2, and Ca^{2+} influx was evaluated by the increase in $[Ca^{2+}]_i$ after addition of extracellular Ca^{2+}. ACh-induced Ca^{2+} influx (nM) in HEK-293 cells was 21 ± 2 in control HEK-293 cells, 52 ± 6 in the cells with PLC-δ1 overexpression, and 75 ± 9 in those with PLC-δ1 overexpression and PKC down-regulation (all $p<0.05$ among 3 groups). Ca^{2+} influx under treatment with nifedipine at 10^{-5}M was 2.9 ± 0.1 times higher in the cells with PLC-δ1 overexpression and 5.6 ± 0.2 times higher in those with PLC-δ1 overexpression and PKC down-regulation compared with the control cells (all $p<0.05$ among 3 groups).
Conclusions: ACh-induced Ca^{2+} influx was enhanced by PLC-δ1 overexpression, but was attenuated by PKC activation. PKC plays an important role in Ca^{2+} influx under enhanced PLC-δ1 in a negative feedback fashion.

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Key words: Coronary spasm; Protein kinase C; Phospholipase C-δ1; Calcium influx; Acetylcholine.

INTRODUCTION

Coronary artery spasm plays an important role in the pathogenesis of not only coronary spastic angina (CSA) [1,2] but the other forms of ischemic heart disease such as myocardial infacrtion [3]. Considering the fact that esophageal motility is enhanced in patients with CSA [4], the presence of a generalized disorder of smooth muscle contraction is strongly suggested.

The underlying mechanisms for coronary spasm are not fully but adequately determined. Apart from the report for alterations of endothelial nitric oxide synthase [5], we have shown the role of phospholipase C (PLC)-δ1 in the pathogenesis of human CSA [6-9]. PLC-δ1 is an isoform of PLC, which is more sensitive to Ca^{2+} than the other isozymes; therefore, the initial increase in Ca^{2+} induced by G protein-linked PLC-β elicits a prolonged activation of PLC-δ1 in a positive feedback fashion (10). PLC-δ1 activity was three times higher in the skin fibroblasts obtained from the CSA patients than that from the control subjects, and its activity was positively correlated with coronary artery vasomotility [6]. A single base variant (864G-A) in the entire coding region of the PLC-δ1 gene, which results in the amino acid replacement of arginine 257 by histidine, was found in 10% of the male CSA patients, and showed an enhanced enzymatic activity [7]. To determine a causative role of enhanced PLC-δ1 activity in CSA, we generated transgenic mice with vascular smooth muscle-specific overexpression of variant PLC-δ1 (TG), and showed ergometrine-induced coronary spasm in this TG [9].

From Department of Cardiology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan

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Extracellular Ca^{2+} entry in coronary vascular smooth muscle cells is regulated by voltage-dependent Ca^{2+} channels and voltage-independent nonselective cation channels. Classical transient receptor potential (TRPC) protein is a kind of nonselective cation channels, which consist of seven members based on their amino acid homology [11]. Within the TRPC family, TRPC3, 6, and 7 are activated by diacylglycerol (DAG) [12]. Although DAG activates PKC and initiates sustained contraction by Ca^{2+}-independent mechanism, the involvement of PKC in Ca^{2+} signaling still remains unclear. Using human embryonic kidney (HEK)-293 cells, we investigated the role of PKC in ACh-induced Ca^{2+} influx under enhanced PLC-δ1 seen in coronary spasm.

MATERIALS AND METHODS

Materials

Fura-2- acetoxymethyl ester (AM) was purchased from Wako Pure Chemical Industries, Ltd., Tokyo, Japan. Ionomycin was from Calbiochem, Darmstadt, Germany. QIA shredder and RNeasy Protect Mini Kit were from QIAGEN, Valencia, CA, USA. Human protein kinase A anchoring protein (AKAP) 5 siRNA, siCONTOROL Non-Targeting siRNA#1 (Cat; D-001210-01), and DharmaFECT Duo transfection reagent (Cat; T-2010-03) were all from Dharmacon Inc., Lafayette, CO, USA. Plasmids of PLC-δ1 and M3 receptor were from Takara Bio Inc. (Kyoto, Japan). TransIT-293 transfection reagent for HEK-293 cells (Cat; T-2010-03) was purchased from Mirus Bio LLC (Madison, WI, USA). Dulbecco's modified Eagle's medium (DMEM) and fatal bovine serum (FBS) were from Gibco, Life Technologies Corporation, Tokyo, Japan. All other reagents were of the finest grade available from Sigma Chemical Co., St. Louis, Missouri, USA.

Cell culture

HEK-293 cells (American Type of Cell Culture) were cultured in DMEM supplemented with 10% FBS at 37oC under 5% CO_2. The cells from the 4th to 7th passages were used for the study. After treatment with various molecules, the viability of the cells, which was determined by trypan blue exclusion, was generally >95%.

Measurement of $[Ca^{2+}]_i$

The cells were subcultured in 6-cm petri dishes, and transfected with both muscarine M3 receptor cDNA and either the human wild type PLC-δ1 or empty vector (all, 1.0 μg DNA/well). After loading with 5 μmol/L fura-2–AM, $[Ca^{2+}]_i$ in response to ACh at 10^{-5} mol/L was measured at excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm as described previously [13]. Calibration was made using ionomycin followed by EGTA-Tris. $[Ca^{2+}]_i$ in response to ACh was also measured in the Ca^{2+}-free medium and addition of extracellular Ca^{2+}. ACh was used since it is widely used for the induction of coronary spasm in patients in Japan [14].

RNA interference

HEK 293 cells were transfected by AKAP5 siRNA or siCONTOROL Non-Targeting siRNA#1 (final concentration 100 nM) at 70-80% confluency by use of transfection reagent, DharmaFECT Duo in the complete medium according to the manufacturer's instructions. This procedure suppressed gene expression by 70-80%.

Statistics

All data are shown as mean ± one SEM. A paired or unpaired t test for comparison of two variables, and one-way ANOVA for multiple comparisons followed by Bonferroni's test were used for statistical analysis. The level of significance was less than 0.05.

RESULTS

Figure 1A illustrates representative waveforms of $[Ca^{2+}]_i$ after the administration of ACh at 10^{-5}M

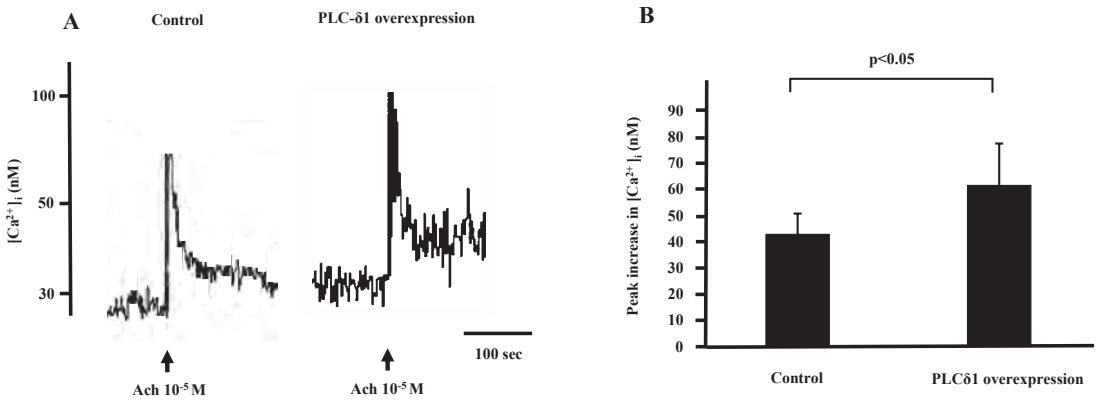

Figure 1. Effect of phospholipase C (PLC)-δ1 overexpression on acetylcholine (ACh)-induced increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) in HEK 293 cells.
(A) Representative waveforms of $[Ca^{2+}]_i$ after the administration of ACh.
(B) ACh-induced peak increase in $[Ca^{2+}]_i$ by PLC-δ1 overexpression (n=4).

in HEK-293 cells. ACh, an inducer of coronary spasm in patients, caused transient and sustained increase in $[Ca^{2+}]_i$ in the cells with and without PLC-δ1 overexpression. As shown in Figure 1B, the peak increase in $[Ca^{2+}]_i$ was higher in the cells with PLC-δ1 overexpression than in those without it ($p<0.05$).

To evaluate the amount of Ca^{2+} influx, the increase in $[Ca^{2+}]_i$ by ACh was examined in Ca^{2+}-free buffer, and after the addition of Ca^{2+} to extracellular space. As shown in Figure 2A, ACh caused transient and small increase in $[Ca^{2+}]_i$ in Ca^{2+}-free buffer, and the addition of extracellular Ca^{2+} resulted in the further increase in $[Ca^{2+}]_i$. This ACh-induced Ca^{2+} influx by adding extracellular Ca^{2+} was augmented in the cells with PLC-δ1 overexpression and/or PKC down-regulation, but attenuated in those with PKC up-regulation. As shown in Figure 2B, the peak $[Ca^{2+}]_i$ influx was enhanced from 21 ± 2 to 52 ± 6 nM by PLC-δ1 overexpression ($p<0.05$). Further activation of PKC with TPA at 10^{-7}M attenuated the peak $[Ca^{2+}]i$ influx (52 ± 6 vs 21 ± 3 nM, $p<0.05$), whereas suppression of PKC with AKAP5 siRNA enhanced it (52 ± 6 vs 75 ± 9 nM, $p<0.05$).

We further investigated the role of PKC in ACh-induced Ca^{2+} influx after treatment with nifedipine at 10^{-5}M. As shown in Figure 3A, ACh also caused transient and small increase in $[Ca^{2+}]_i$ in Ca^{2+}-free buffer, and the addition of extracellular Ca^{2+} resulted in the further increase in $[Ca^{2+}]_i$. This ACh-induced Ca^{2+} influx by adding extracellular Ca^{2+} was augmented in the cells with PLC-δ1 overexpression, and was further increased in those with PLC-δ1 overexpression and PKC down-regulation. As shown in Figure 3B, Ca^{2+} influx under treatment with nifedipine at 10^{-5}M was 2.9 ± 0.1 times higher in the cells with PLC-δ1 overexpression and 5.6 ± 0.2 times higher in those with PLC-δ1 overexpression and PKC down-regulation compared with the control cells (all $p<0.05$ among 3 groups).

DISCUSSION

The major findings of this study were as follows. ACh-induced Ca^{2+} influx in HEK-293 cells was enhanced by PLC-δ1 overexpression and/or PKC down-regulation, but attenuated by PKC up-regulation. ACh-induced Ca^{2+} influx under treatment with nifedipine was higher in the cells with PLC-δ1 overexpression and/or

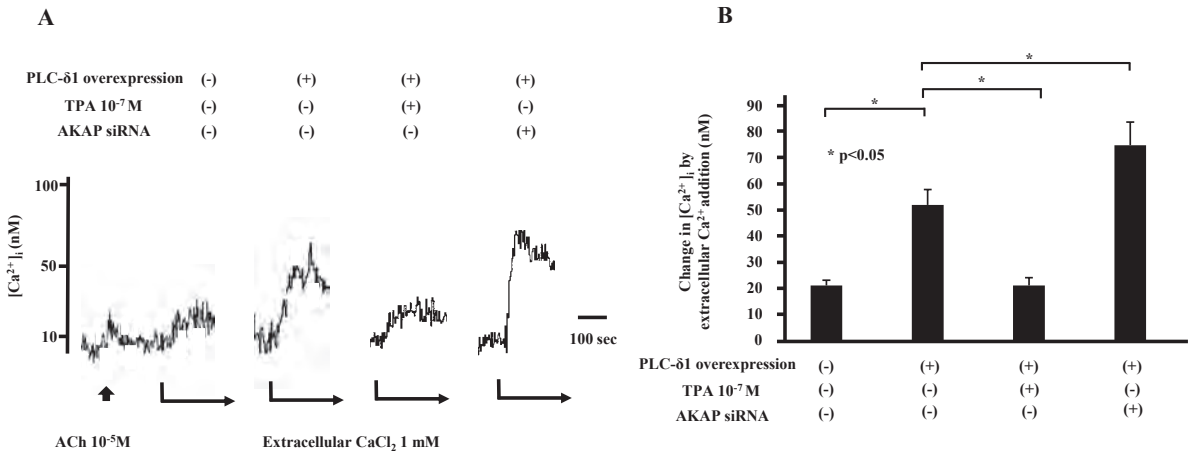

Figure 2. Effect of phospholipase C (PLC)-δ1 overexpression, TPA, and AKAP5 siRNA on acetylcholine (ACh)-induced Ca^{2+} influx in HEK 293 cells.
(A) Representative waveforms of $[Ca^{2+}]_i$ in Ca^{2+}-free buffer and after the addition of extracellular Ca^{2+}.
(B) Change in $[Ca^{2+}]_i$ by extracellular Ca^{2+} addition in PLC-δ1 overexpression, TPA, and AKAP5 siRNA (n=4).

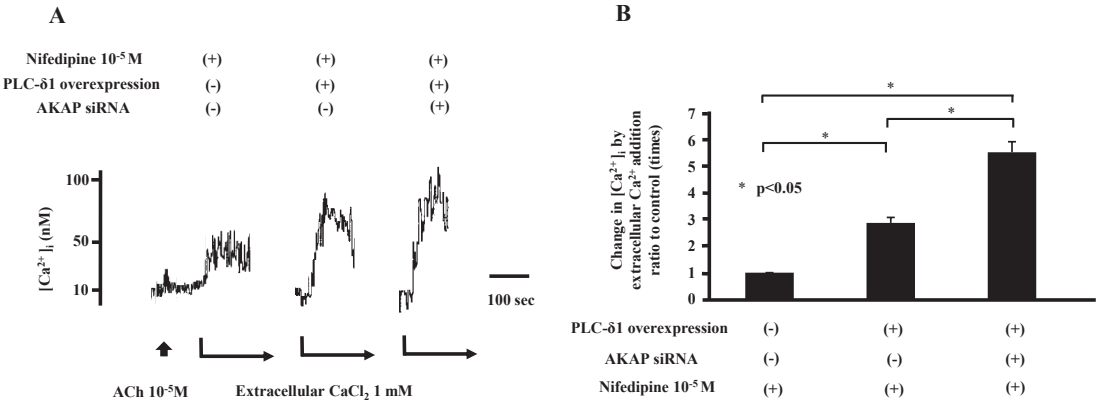

Figure 3. Effect of phospholipase C (PLC)-δ1 overexpression and AKAP5 siRNA on acetylcholine (ACh)-induced Ca^{2+} influx under treatment with nifedipine at 10^{-5}M in HEK-293 cells.
(A) Representative waveforms of $[Ca^{2+}]_i$ in Ca^{2+}-free buffer and after the addition of extracellular Ca^{2+}.
(B) Change in $[Ca^{2+}]_i$ by extracellular Ca^{2+} addition (ratio to control) in PLC-δ1 overexpression and AKAP5 siRNA (n=4).

PKC down-regulation compared with the control cells.

By using the HEK293 cells transfected with muscarine M3 receptor, PLC-δ1, or AKAP5 siRNA, we investigated the role of PKC in the pathogenesis of CSA. We first confirmed that PLC-δ1 overexpression enhanced the $[Ca^{2+}]_i$ response to ACh which consists of mobilization of Ca^{2+} from intracellular Ca^{2+} store and Ca^{2+} influx. In HEK-293 cells, PLC activity was enhanced approximately by two times compared with those without transfection of PLC-δ1 [7], and it was comparable to the activity of PLC-δ1 seen in CSA patients [6], suggesting

the suitability of this study to analyze the pathogenesis of human CSA. We used ACh that binds muscarine M3 receptor linking to the Gq–PLC-β pathway, because its compound is widely used as a provocation tests for coronary spasm in the clinical setting in Japan [14]. In the cells transfected with PLC-δ1, in addition to the enhanced peak increase in $[Ca^{2+}]_i$ and prolonged sustained phase, the baseline level of $[Ca^{2+}]_i$ was elevated. Increased basal level of $[Ca^{2+}]_i$ as well as hyper-responsiveness to ACh seems reasonable to explain the pathogenesis of CSA; the basal vascular tone and the vasoconstrictor response to the diverse stimuli were enhanced [15-17]. We previously demonstrated that PLC activity was positively correlated not only with basal coronary artery tone but with the maximal and averaged constrictor responses of the coronary artery to ACh [6].

PLC produces inositol 1,4,5-trisphosphate (IP_3) and DAG by hydrolyzing phosphatidyl inositol 4,5- bisphosphate. IP3 mobilizes Ca^{2+} from the intracellular stores and elicits rapid contraction of the vascular smooth muscle cells, whereas DAG activates PKC and initiates sustained contraction by Ca^{2+}-independent mechanism. DAG also activates TRPC channel and induces rapid and sustained constriction by Ca^{2+}-dependent mechanism which consists of nonselective cation influx and the following activation of voltage-gated Ca^{2+} channels [12]. When ACh was added in Ca^{2+}-free buffer, the transient phase of $[Ca^{2+}]_i$ was still greater in the cells transfected with PLC-δ1 than those without PLC-δ1, and the sustained phase was abolished in both kinds of cells. These suggest that PLC-δ1-induced enhancement of Ca^{2+} signal was dependent on a rapid transient increase of mobilization from intracellular Ca^{2+} store and a sustained phase of extracellular Ca^{2+} entry.

Because TRPC may be down-regulated by PKC in a negative feedback mechanism [18], we examined the role of PKC in Ca^{2+} influx in HEK-293 cells with PLC-δ1 overexpression. The result showed that ACh-induced Ca^{2+} influx by adding extracellular Ca^{2+} was augmented in the cells with PLC-δ1 overexpression, and further increased in those with PLC-δ1 overexpression and PKC down-regulation. Thus, ACh-induced Ca^{2+} entry seems to be regulated by either DAG-induced facilitation or PKC-induced suppression.

The present study first showed that under enhanced PLC-δ1 seen in coronary spasm, PKC suppressed ACh-induced Ca^{2+} influx. Thus, PKC seems to play an important role in Ca^{2+} influx under enhanced PLC-δ1 in a negative feedback fashion. However, the precise underlying mechanism still remains unclear, and the further study is needed.

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