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AHNAK-mediated Activation of Phospholipase C-γ1 through Protein Kinase C*

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We have recently shown that phospholipase C-γ (PLC-γ) is activated by the central repeated units (CRUs) of the AHNAK protein in the presence of arachidonic acid. Here we demonstrate that four central repeated units (4 CRUs) of AHNAK act as a scaffolding motif networking PLC-γ and PKC-α. Specifically, 4 CRUs of AHNAK bind and activate PKC-α, which in turn stimulates the release of arachidonic acid near where PLC-γ1 is localized. Moreover, 4 CRUs of AHNAK interacted with PLC-γ and the concerted action of 4 CRUs with arachidonic acid stimulated PLC-γ activity. Stimulation of NIH3T3 cells expressing 4 CRUs of AHNAK with phorbol 12-myristate 13-acetate resulted in the increased generation of total inositol phosphates (IP_T) and mobilization of the intracellular calcium. Phorbol 12-myristate 13-acetate-dependent generation of IP_T was completely blocked in NIH3T3 cells depleted of PLC-γ1 by RNA interference. Furthermore, bradykinin, which normally stimulated the PLC-β isozyme resulting in the generation of a monophasic IP_T within 30 s in NIH3T3 cells, led to a biphasic pattern for generation of IP_T in NIH3T3 cells expressing 4 CRUs of AHNAK. The secondary activation of PLC is likely because of the scaffolding activity of AHNAK, which is consistent with the role of 4 CRUs as a molecular linker between PLC-γ and PKC-α.

AHNAK, a nuclear phosphoprotein with the estimated molecular mass of 700 Da, was originally identified in human neuroblastomas and skin epithelial cells (1–3). AHNAK contains three distinct structural regions: the NH_2-terminal 251-amino acid region, a large central region of about 4300 amino acids with 36 repeated units, and the COOH-terminal 1002 amino acids region. The carboxyl-terminal region of AHNAK proteins was reported to play an important role in cellular localization and in interaction with L-type Ca^{2+} channels in cardiac cells (4) and with the calcium-binding S100B protein in rat embryo fibroblast cells (5). In low calcium concentrations, AHNAK proteins are mainly localized in the nucleus, but the increase in intracellular calcium levels leads the protein to translocate to plasma membrane (3). Phosphorylation of serine 5535 in the carboxyl-terminal AHNAK protein by nuclear PKB[1] was shown to be essential for its export from the nucleus (6). The central repeated unit in AHNAK is 128 amino acids in length and displays a heptasequence motif, (D/E)$\phi\Omega\phi$K(A/G)P, where ϕ and Ω represent hydrophobic and hydrophilic amino acid residues, respectively. Shtivenman *et al.* (1, 2, 6) suggested that this sequence exists as a β-strand and polyionic rod with hydrophobic amino acids facing inward and hydrophilic amino acids facing outward. It is suggested that the central repeats likely support the structural integrity of AHNAK (1, 2, 6).

Activation of phosphoinositide-specific phospholipase C (PLC) is a key event in cellular signal transduction involved in cell growth, proliferation, metabolism, and secretion (7). PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol (1,4,5)-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). To date, a total of 11-different isozymes of PLC have been identified in mammalian cells, and these can be classified into four subfamilies, β (β1-β4), γ (γ1 and γ2), δ (δ1–δ4), and ϵ isozymes. Based on their primary structures, this classification has been correlated with their different activation mechanisms (7). Although protein-tyrosine kinase (PTK)-mediated PLC-γ isozyme activation is well established, lipid-derived second messengers such as phosphatidic acid, phosphatidylinositol (3,4,5)-triphosphate, and arachidonic acid (AA) were also proposed to activate the isozyme (7). Furthermore, concerted action of arachidonic acid with tau or with repeated units of AHNAK was also shown to stimulate the activation of PLC-γ isozymes (8, 9).

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[1] The abbreviations used are: PKB, protein kinase B; PLC, phospholipse C; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol (1,4,5)-trisphosphate; DAG, 1,2-diacylglycerol; PTK, protein-tyrosine kinase; AA, arachidonic acid; MAPK, mitogen-activated protein kinase; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; PMA, phorbol 12-myristate 13-acetate; GFP, green fluorescent protein; PDGF, platelet-derived growth factor; HA, hemagglutinin; GST, glutathione *S*-transferase; TRITC, tetramethylrhodamine isothiocyanate; siRNA, small interfering RNA; CRU, central repeated unit; BK, bradykinin; DMEM, Dulbecco's modified Eagle's medium; IP_T, total inositol phosphate; PBS, phosphate-buffered saline; $[Ca^{2+}]_i$, intracellular Ca^{2+}; SH3, Src homology domain 3.

The 11 PKC isozymes thus far identified display diverse tissue expression, subcellular localization, cofactor requirements, and functional diversity (10–13). The PKC isozymes can be classified into three groups according to their regulatory properties, which are in turn governed by the presence of specific domains in the proteins. The conventional PKCs include PKCα, βI, βII, and γ, and these isoforms can be activated by Ca^{2+} and/or by DAG and phorbol esters. The novel PKCs, δ, ϵ, θ, and η, can also be activated by DAG and phorbol esters but are Ca^{2+}-independent. The atypical PKCs, which include PKCξ and PKCι, are unresponsive to Ca^{2+} and DAG/phorbol esters. It has been established that PKC isozymes activate the Raf-MAPK cascade and NF-κB as downstream molecules in cell signaling (14).

Phospholipase A_2 (PLA_2) enzymes hydrolyze fatty acid from the *sn*-2 position of phospholipid with the concomitant production of lysophospholipid. Mammalian cells contain structurally diverse forms of PLA_2 including secretory PLA_2, calcium-independent PLA_2, and the 85-kDa cytosolic PLA_2 ($cPLA_2$). It has been reported that AA is produced in response to diverse stimuli including interleukin-1, tumor necrosis factor, epidermal growth factor, okadaic acid, the phagocytic particle zymosan, and phorbol 12-myristate 13-acetate (PMA) (15–17). These reports indicate that AA, a product of PLA_2, plays the role not only of an important initiator of inflammatory processes but also of a regulator of signaling process (16).

Although 4 central repeated units (CRUs) of the AHNAK protein bind and activate PLC-γ *in vitro*, the cellular function of the CRUs in AHNAK is not clear. Our results suggest that the 4 CRUs of AHNAK concomitantly interact with PKC-α and PLC-γ in response to PMA. It is likely that PKC-α in a ternary complex translocates to the membrane and then induces the release of AA through $cPLA_2$ activation. Once released, AA likely activates PLC-γ through a concerted action with AHNAK. Taken together, these results indicate that 4 CRUs of AHANK act as a scaffolding protein networking for PLC-γ and PKC-α.

EXPERIMENTAL PROCEDURES

Materials—[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (189 Ci/mmol) and *myo*-[2-^{3}H]inositol were purchased from PerkinElmer Life Sciences. GF109203X, PMA, bradykinin (BK), and AG1478 were purchased from Calbiochem. Fluo-4/AM was obtained from Molecular Probes. MACSelect K^K MicroBeads were obtained from Miltenyi Biotec, and SuperFect was purchased from Qiagen. The anti-HA, anti-PKC-β3, and anti-PKC-α monoclonal antibodies were purchased from Roche Diagnostics, Santa Cruz Biotechnology, and Upstate Biotechnology, respectively.

Cell Cultures—NIH3T3 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. COS-7 and TV1 null cells were cultured in DMEM supplemented with 10% fetal bovine serum.

Plasmids—Human AHNAK cDNA was obtained by screening the human male BAC library. The division of AHNAK into N (amino acid residues 1–257), C1 (amino acid residues 4640–5386), and C2 (amino acid residues 5258–5643) domains was based on a previously published report (4). The cDNA fragments were amplified by PCR and inserted into the pcDNA3-HA vector by digestion with EcoRI and XhoI. The gene for 4 CRUs of AHNAK (amino acid residues 3860–4412) was subcloned into pcDNA3-HA by digestion with EcoRI and XhoI. The gene for 4 CRUs of AHNAK was also obtained by PCR and inserted into pEGFP-N1 and pDsRed-C1 (Clontech) by digestion with XhoI and HindIII and HindIII and BamHI, respectively. All constructs were checked by restriction site mapping and sequencing.

Transfection—NIH3T3 cells were plated at a density of 1.5×10^5 cells/well in six-well plates. The cells were transfected with 4 μg of pcDNA3, pcDNA3-HA-AHNAK-N, pcDNA3-HA-4CRUs, pcDNA3-HA-C1, pcDNA3-HA-C2, pDsRed-C1, or pDsRed-C1–4CRUs using SuperFect reagent according to the manufacturer's protocol and maintained in the completed medium for 24 h. The cells were serum-starved for 12 h and then stimulated with various agonists for the indicated times.

Release of Arachidonic Acid—NIH3T3 cells (2.5×10^5) were plated at 6-well plates. The cells were cultured for 24 h and labeled by incubation for 16 h in 1 ml of serum-free medium containing [^{3}H]arachidonic acid (0.5 μCi/ml) and 0.1% fatty acid-free bovine serum albumin. The cells were then washed twice with DMEM containing 0.1% bovine serum albumin and incubated with 100 nM PMA in the absence or presence of 10 μM A23187 for 10 min. Radioactivity in the supernatant fractions and the cell lysates containing 1% Triton X-100 were measured by liquid scintillation counting. The percentage of arachidonic acid release was calculated as the (medium cpm/(cells + medium) cpm) × 100 and then normalized to the value of unstimulated controls (18, 19).

Measurement of Total Inositol Phosphate (IP_T) in Cells—NIH3T3 cells were labeled with inositol-free DMEM supplemented with *myo*-[2-^{3}H]inositol (1 μCi/ml, 25 mCi/mmol) (DuPont Biotechnology Systems) for 14 h and then washed with inositol-free DMEM (20). The cells were subsequently incubated in DMEM containing 20 mM LiCl for 30 min and stimulated with 100 nM PMA for 10 min or stimulated with 1 μM bradykinin for the indicated time. To inhibit PKC activity, the cells were treated with 5 μM GF109203X for 10 min followed by 100 nM PMA for 10 min. To inhibit Src and epidermal growth factor receptor activity, the cells were pretreated with 10 μM PP-1, 1 μM AG1478 for 30 min and then stimulated with 1 μM bradykinin for the indicated time periods. The incubation was terminated by adding perchloric acid to a final concentration of 5% (w/v). The cells were scraped into Eppendorf tubes and centrifuged at 15,000 × *g* for 20 min at 4 °C. The supernatant was equilibrated with 2 M KOH, 1 mM EDTA and applied to a SAX column connected to a high performance liquid chromatograph (Hewlett Packard series 1100). Bound inositol phosphates were eluted by applying a linear gradient (0–1.0 M ammonium phosphate) at a flow rate of 2 ml/min. Radioactivity in the resulting fraction, corresponding to liberated [^{3}H]IP_T, was measured using a liquid scintillation counter.

Magnetic Enrichment of NIH3T3 Cells Expressing AHNAK—NIH3T3 cells were co-transfected pMACS K^K with pcDNA3-HA or pcDNA3-HA-4CRUs using FuGENE 6 (Roche Diagnostics) as described in the manufacturer's protocol. After 24 h incubation, the cells were washed with phosphate-buffered saline (PBS) without EDTA. The cells were again incubated with 500 μl of trypsin solution per 100-mm dish until dissociated from culture dish and from each other. Trypsinization was stopped by adding 100 μl of 100% fetal bovine serum. The cells were then incubated with 80 μl of MACSelect K^K MicroBeads per 100-mm dish for 15 min at room temperature and PBS containing 2 mM EDTA and 0.5% fetal bovine serum was added to a final volume of 2 ml. The cells expressing MACS K^K protein were separated from magnetic columns, plated on 6-well plates, and allowed to recover for 24 h. The cells were subsequently incubated for 18 h in inositol-free DMEM supplemented with *myo*-[^{3}H]inositol (1.5 μCi/ml, 25 mCi/mmol) (DuPont), and processed as described above.

Measurement of Intracellular Calcium ($[Ca^{2+}]_i$)—$[Ca^{2+}]_i$ was measured using a laser scanning confocal microscope (21). NIH3T3 cells were grown on coverslips and transfected with pDsRed-C1–4CRUs. Transfected or nontransfected NIH3T3 cells were serum-starved for 12 h, incubated with 2 μM Fluo-4/AM in serum-free medium for 40 min, and washed three times with Ca^{2+}-free Locke's solution (158.4 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES buffer adjusted to pH 7.3, 10 mM glucose, and 0.2 mM EGTA). The coverslips containing the stained cells were mounted on a perfusion chamber (21) and subjected to confocal laser scanning microscopic analysis (Olympus LV300). Prior to observing the release of real calcium in response to PMA, NIH3T3 cells expressing red fluorescence protein-tagged AHNAK were pre-selected through scanning with 543- and 560-nm emission filters and scanned every second with a 488-nm excitation argon laser and a 515-nm long pass emission filter. A solution containing 100 nM PMA was then added to the cells with an automatic pumping system (21). About 150 images resulting from the scanning were analyzed for changes of $[Ca^{2+}]_i$ at a single cell level. The results were expressed as relative fluorescence intensity.

Construction of Small Interfering RNA (SiRNA) for PLC-γ1—Specific sequences of 19-nucleotide sequence (**ctactactctgaggagacc**, residues 1695 to 1713) of the human PLC-γ1 cDNA were selected for synthesis of a siRNA. pSUPER vector for siRNA was purchased from Oligoengine (22). The phosphorylated oligonucleotides were annealed and cloned into the pSUPER vector with BglII (5′ end) and HindIII (3′ end). Cells were transfected with the resulting construct and cultured for 48 h in complete medium. The transfected cells were deprived of serum for 12 h, incubated for 10 min in the absence or presence of PMA or for the indicated times in the absence or presence of bradykinin, and then analyzed by measurement of total inositol phosphates. The depletion of endogenous PLC-γ1 by the siRNA was confirmed by immunoblot analysis.

Immunofluorescence—COS-7 cells were grown on coverslips and transfected with pEGFP-N1–4CRUs. The cells were serum-starved for 12 h, stimulated with 100 nM PMA for 10 min, washed with cold PBS, fixed with 3.5% paraformaldehyde in PBS for 10 min at room temperature, and permeabilized in 0.5% Triton X-100. Nonspecific sites were blocked by treating the cells with PBS containing 0.05% gelatin and 0.5% bovine serum albumin for 1 h. The cells were incubated with primary antibodies against PKC-α or PLC-γ in PBS for 1 h at room temperature, washed with PBS, cells were incubated with the secondary antibody (TRITC-conjugated goat anti-mouse-IgG), and then mounted on glass slides using a drop of Aqua-Poly/mount. Images were recorded using a confocal laser scanning microscope (Carl Zeiss 510).

GST Fusion Protein Binding Assays—Cultures of *Escherichia coli* BL21 containing pGEX4T1 and pGEX4T1–4CRUs were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 30 °C. The harvested bacteria were suspended in PBS containing 1% Triton X-100 and protease inhibitors (0.1 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) and lysed by sonication. After centrifugation at 15,000 $\times g$ for 20 min, the supernatant was incubated with glutathione-agarose beads for 3 h at 4 °C. The samples were washed three times with PBS containing 1% Triton X-100 and subjected to immunoblot analyses.

Immunoprecipitation and Immunoblotting—Lysates (1–2 $\times$ 10^6 cells) were mixed with antibodies (0.5–1 μg) for 4 h, followed by addition of 40 μl of protein G-Sepharose for 2 h at 4 °C. Immune complexes were washed five times with lysis buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 0.1 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 10% glycerol). After boiling 2 times in sample buffer, samples were subjected to SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were immunoblotted with the indicated primary antibodies, followed by horseradish peroxidase-conjugated goat secondary antibodies. Bands were visualized by chemiluminescence.

RESULTS

PMA Stimulates PLC-γ Activation in NIH3T3 Cells Expressing Four Central Repeated Units of AHNAK—In a previous report (9), we have shown that AHNAK binds and activates PLC-γ in the presence of AA. To verify the cellular function of the AHNAK protein as an activator of PLC-γ, we first identified an agonist that can generate AA in cells. It is known that PMA, a known PKC activator, can activate cPLA$_2$ and then release AA in various cell types (23). NIH3T3 cells were labeled with [^{3}H]AA, and the cPLA$_2$ activity was monitored by measuring the release of [^{3}H]AA to the supernatant in the absence or presence of PMA stimulation. Stimulation of the cell with PMA resulted in the maximal release of [^{3}H]AA within 30 min followed by a gradual decrease to basal level (Fig. 1).

To determine whether AHNAK can activate PLC-γ in response to PMA, we transiently expressed the hemagglutinin (HA)-tagged AHNAK (HA-AHNAK) derivative in NIH3T3 cells and measured the production of IP$_T$ as an indicator of PLC activity. The large size of AHNAK makes it virtually impossible to express the full-length of the protein, AHNAK was divided into three domains based on sequence analysis and functional studies previously reported (4, 9); HA-N (amino acids 1–257), HA-4CRUs (amino acids 3859–4412), HA-C1 (amino acids 4640–5386), and HA-C2 (amino acids 5258–5643) (Fig. 2*A*). We found that PMA stimulated IP$_T$ production in cells expressing HA-4CRUs by 150% but not in those expressing other regions of AHNAK (Fig. 2*B*). Moreover, inhibition of PKC activity by pretreatment of the cells with GF109203X abrogated production of IP$_T$ in cells expressing HA-4CRUs. These results suggest that PKC might be involved in the AHNAK-dependent activation of PLC. The rather modest level increase in IP$_T$ generation by PMA in NIH3T3 cells is mostly likely because HA-4CRUs transfection efficiency is low. To confirm the effect of AHNAK on PLC activation, we transfected NIH3T3 cells with HA-4CRUs and pMACS K^K, which encodes truncated mouse H-2K^k protein, thus allowing enrichment of cells expressing HA-4CRUs using the MACSelect K^K magnetic bead. Incubation of the selected cells expressing the HA-4CRU protein with PMA resulted in an increase of IP$_T$ generation by 7-fold (Fig. 2*D*).

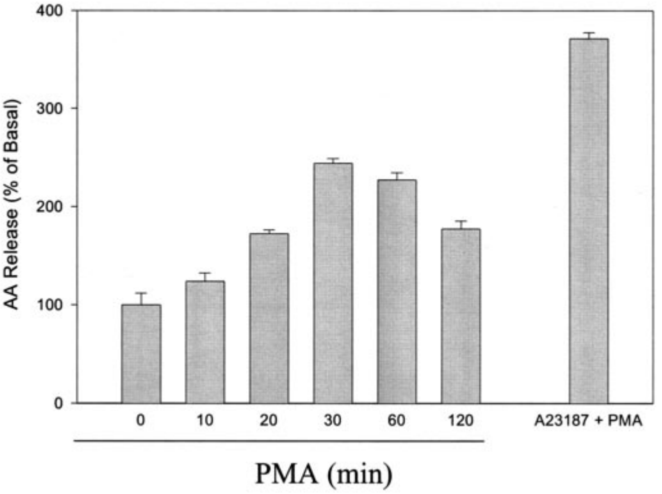

FIG. 1. **NIH3T3 cells release arachidonic acid after PMA treatment.** NIH3T3 cells were labeled with [^{3}H]arachidonic acid for 14 h, and washed and treated with 100 nM PMA for the various times. Radioactivity was determined by scintillation counting. The percentage of [^{3}H]arachidonic acid released in each case is normalized to the value obtained with controls. Results (average ± S.E.) are representative of three independent experiments.

We next examined whether PMA can trigger intracellular Ca^{2+} mobilization ([Ca^{2+}]$_i$) in NIH3T3 cells expressing 4 CRUs of AHNAK. NIH3T3 cells were transfected with RFP-4CRU and the intracellular Ca^{2+} concentration was measured by laser-based confocal microscopy with Fluo-4 dye. PMA failed to mobilize intracellular calcium in untransfected cells, whereas stimulation of NIH3T3 cells expressing RFP-4CRU with PMA resulted in a rapid and transient increase of [Ca^{2+}]$_i$ (Fig. 2*E*). These results suggest that AHNAK is involved in production of IP$_T$ and mobilizes intracellular Ca^{2+} in response to PMA.

To prove the selectivity for PLC-γ1 in AHNAK-mediated IP$_T$ generation, we subjected NIH3T3 cells to transient transfection with pSUPER-PLC-γ1 encoding a siRNA specific for the PLC-γ1 gene (22). The cells transfected with the siRNA vector exhibited a marked reduction in the abundance of the endogenous PLC-γ1 protein compared with cells transfected with the empty pSUPER vector (Fig. 3*A*). NIH3T3 cells transfected with the pSUPER-PLC-γ1 vector failed to generate IP$_T$ in response to PMA, whereas the cells transfected with pSUPER alone exhibited a marked increase of IP$_T$ generation in response to PMA stimulation, suggesting that generation of IP$_T$ results from AHNAK-mediated activation of PLC-γ1 (Fig. 3*A*). The central role of PLC-γ1 in AHNAK-mediated IP$_T$ generation was further demonstrated in PLC-γ1-null fibroblasts (TV1-null) (24). Expression of 4 CRUs of AHNAK in PLC-γ1-null fibroblasts failed in IP$_T$ generation in response to PMA, whereas add-back expression of PLC-γ1 in the fibroblast cells expressing 4 CRUs of AHNAK restored PMA-dependent IP$_T$ generation (Fig. 3*B*). These results demonstrate that the PLC-γ1 isozyme plays the central role in PMA-induced IP$_T$ generation in the presence of AHNAK.

Central Repeated Units of the AHNAK Protein Interact with PLC-γ in Response to PMA—We previously demonstrated a direct interaction between AHNAK and PLC-γ1 in an AA-dependent manner *in vitro* but did not establish whether PMA can induce 4 CRUs of the AHNAK-PLC-γ1 complex formation in cells. To address this question, we performed co-immunoprecipitation experiments with monoclonal antibodies against HA. Cells expressing the HA-4CRUs protein were either unstimu-

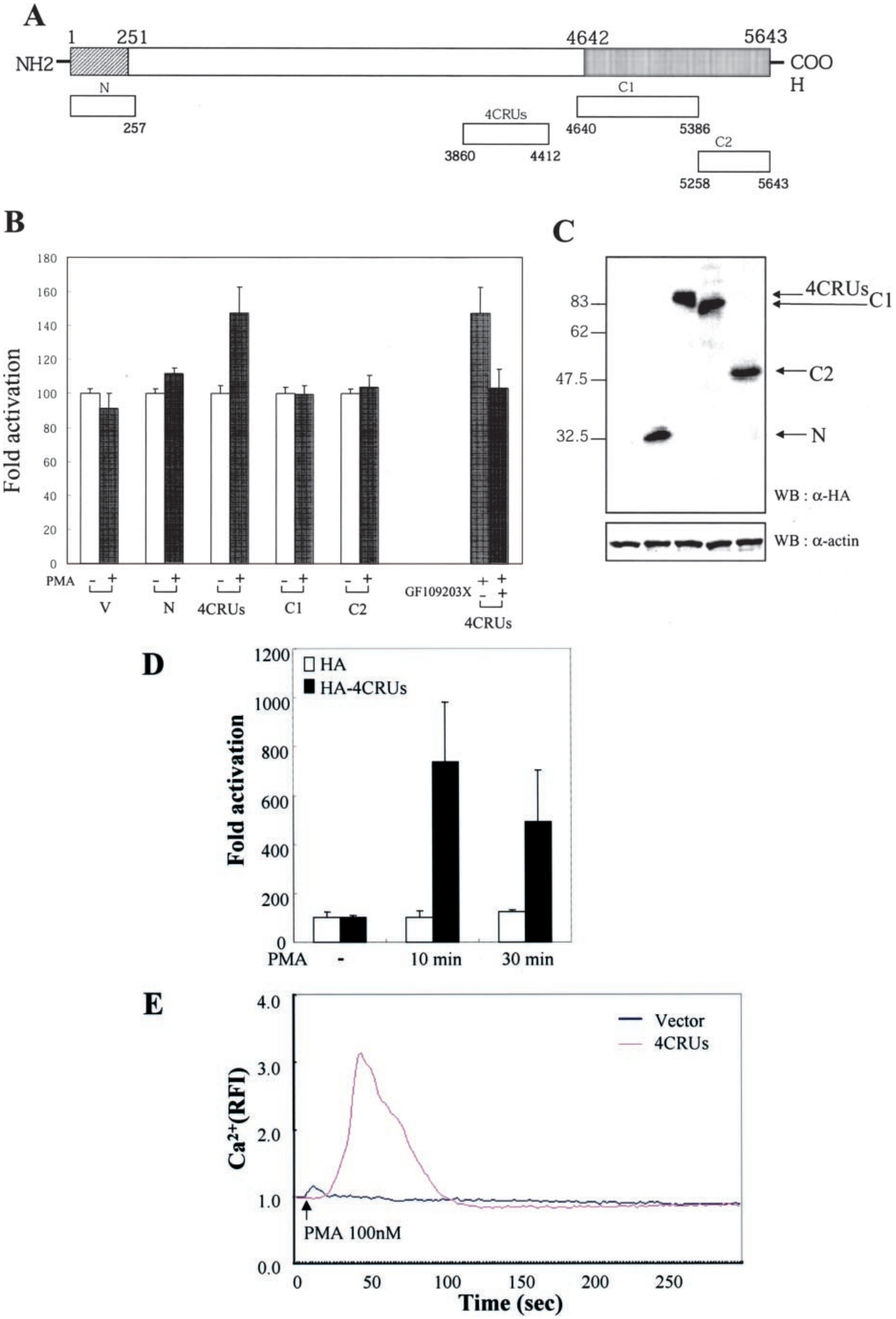

FIG. 2. **PMA augments the production of inositol phosphates and intracellular calcium mobilization in NIH3T3 cells expressing 4 CRUs of AHNAK.** *A*, schematic linear representation of the HA-tagged AHANK constructs. *B*, NIH3T3 cells were transfected with vectors, HA-N, -C1, -C2, and -4CRUs DNA, respectively. Cells were stimulated with PMA, and IP_T generated was measured as described under

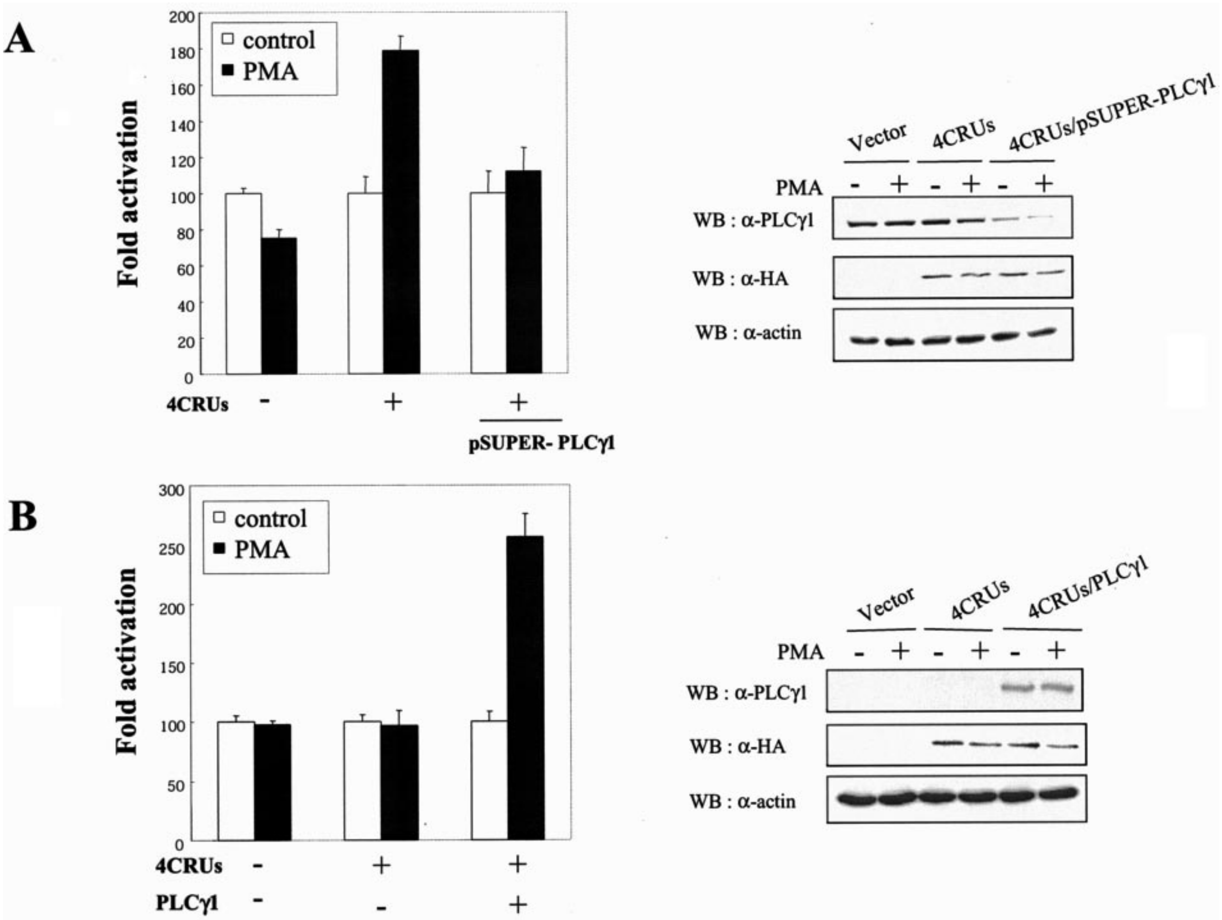

FIG. 3. **PLC-γ1 is required for the generation of AHNAK-mediated total inositol phosphates responding to PMA.** *A*, NIH3T3 cells were transfected with either pSUPER-PLC-γ1 encoding PLC-γ1-specific siRNA and HA or HA-4CRU. Control cells were transfected with pSUPER and pcDNA3 (HA). After culture for 36 h, the cells were labeled with inositol-free DMEM supplemented with *myo*-[2-^{3}H]inositol for 14 h and stimulated with PMA. IP$_T$ generation by PLC-γ1 was measured as described under "Experimental Procedures." Data are mean ± S.E. of values from three independent experiments. Cell lysates were blotted with the indicated antibodies. *B*, empty vector (pcDNA) or HA-tagged 4 CRUs with or without pcDNA3.1-PLC-γ1 were coexpressed in PLC-γ1-null mouse embryonic fibroblasts (TV1-null cells). After culture for 36 h, cells were stimulated with PMA. IP$_T$ generation as a PLC-γ1 activity was measured as described under "Experimental Procedures." Data are mean ± S.E. of values from three independent experiments. Cell lysates were blotted with the indicated antibodies. *WB*, Western blot.

lated or stimulated with PMA for 10 min and then lysed in a buffer containing nonionic detergent. The lysates were immunoprecipitated with an anti-HA monoclonal antibody and examined by immunoblotting with monoclonal antibodies against PLC-γ1, PLC-β1, PLC-β3, and PLC-δ1, respectively. PLC-β3 is the predominantly expressed PLC-β isozyme in NIH3T3 cells. The 4 CRUs of the AHNAK protein interacted with PLC-γ in a PMA-dependent manner, whereas other isozymes failed to interact with AHNAK (Fig. 4*A*). To investigate whether platelet-derived growth factor (PDGF) stimulates the interaction of 4 CRUs of AHNAK with PLC-γ1, NIH3T3 cells expressing the HA-4CRU protein were either unstimulated or stimulated with PDGF for 10 min and then lysed in a buffer containing nonionic detergent. The lysates were immunoprecipitated with antibodies against HA and examined by immunoblotting with monoclonal antibodies against PLC-γ1. The result indicates that 4 CRUs of the AHNAK protein failed to interact with PLC-γ1 in response to PDGF suggesting that AHNAK mediates PTK-independent PLC-γ activation (Fig. 4*B*).

Moreover, we have observed colocalization of PLC-γ1 with AHNAK in response to PMA. Endogenous PLC-γ and GFP-4CRU were located in the cytoplasm and perinuclear regions in resting cells, whereas GFP-4CRU was co-localized with PLC-γ in plasma membrane (*arrow*) and the perinuclear region in response to PMA stimulation (Fig. 4*C*). *Asterisks* indicate NIH3T3 cells untransfected with 4 CRUs of AHNAK. Plasma membrane localization of PLC-γ in response to PMA was not observed in untransfected NIH3T3 cells (indicated by *asterisks*). Results from immunoprecipitation experiments and confocal microscopy indicate that PMA induces formation of the AHNAK-PLCγ complex in the plasma membrane.

Central Repeated Units of AHNAK Protein Interact with PKC Isozyme—Because NIH3T3 cells appear to mainly express the PKC-α isozyme (25), we employed co-immunoprecipitation ex-

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"Experimental Procedures." *C*, cell lysates were subjected to immunoblot analysis with antibodies to HA; the membrane was re-probed with antibodies to actin. *D*, NIH3T3 cells were transfected with pMACS K^K with vector or HA-4CRU. After 24 h incubation, the transfected cells were enriched by AutoMACS, stabilized for 12 h, and stimulated with PMA. PLC-γ1 activity was measured as described under "Experimental Procedures." Data are expressed as the percentage of the value for pcDNA3 (HA)-transfected cells with PMA and represent the average ± S.E. from three independent experiments. *E*, PMA stimulates mobilization of intracellular calcium in NIH3T3 cells expressing 4 CRUs of AHNAK. Serum-starved NIH3T3 cells overexpressing pDsRed-C1 and pDsRed-C1–4CRU were labeled with 2 μM Fluo-4/AM for 40 min, stimulated with 100 nM PMA in the Ca^{2+}-free Lockes' solution, and [Ca^{2+}]$_i$ was monitored using a laser scanning confocal microscope as explained under "Experimental Procedures." Results are expressed as the relative fluorescence intensity. Each trace is a single cell representative from at least four separate experiments. *WB*, Western blot.

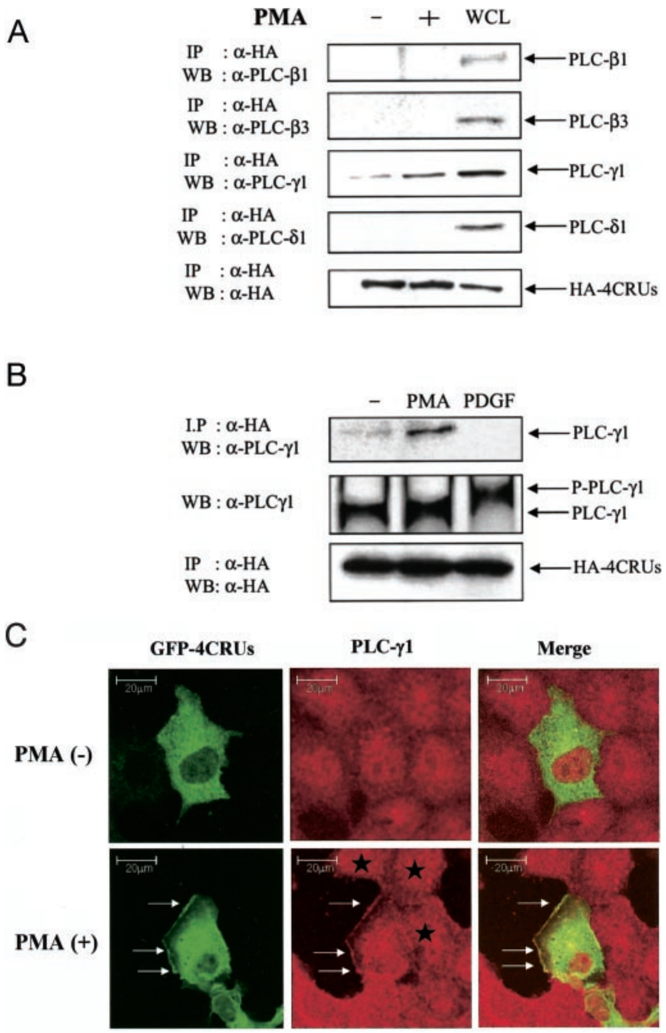

FIG. 4. **4 CRUs of AHNAK interact with only PLC-γ.** NIH3T3 cells were transfected with pcDNA3-HA and pcDNA3-HA-4CRU, and treated with 100 nM PMA or 100 ng/ml PDGF for 10 min. *A*, the cell lysates were immunoprecipitated with anti-HA and then analyzed by immunoblotting using anti-PLC-β1, -β3, -γ1, and -δ1. Then, the membrane was re-probed and immunoblotted using anti-HA. *WCL* means whole cell lysates of NIH3T3. *B*, cell lysates were immunoprecipitated with anti-HA and then analyzed by immunoblotting with antibodies to PLCγ1 and HA. *C*, NIH3T3 cells expressing GFP-4CRU were unstimulated (*upper panel*) or stimulated with 100 nM PMA for 10 min (*lower panel*), fixed, and stained with antibody against PLC-γ. TRITC-conjugated goat anti-mouse IgG was used as a secondary antibody for staining PLC-γ. *Asterisks* indicate NIH3T3 cells not transfected with 4 CRUs of AHNAK. *WB*, Western blot.

periments and confocal microscopy experiments to investigate molecular interaction between 4 CRUs of AHNAK and PKC-α. We have observed that PKC-α interacts with 4 CRUs of AHNAK in response to PMA stimulation in a GST pull-down assay and co-immunoprecipitation experiments (Fig. 5, *A* and *B*). To examine co-localization of 4 CRUs of AHNAK with PKC-α in cells, we performed confocal microscopy with cells expressing GFP-4CRUs. PKC-α was stained with antibody against PKC-α. GFP-4CRUs and PKC-α were dispersed in the cytoplasm in the absence of PMA, whereas GFP-4CRU (*arrow*) was co-localized with endogenous PKC-α (*arrow*) in plasma membrane upon PMA stimulation (Fig. 5*C*).

Several lines of evidence suggest that PMA induces phosphorylation of Ser^{1248} in PLC-γ1 (26). For example, PKC desensitized the PLC-γ1 activity upon OKT-3 activation via Ser^{1248} phosphorylation in Jurkat T cells (26). To elucidate the role of PKC in the complex, we investigated the effect of 4 CRUs on Ser^{1248} phosphorylation of PLC-γ1 in response to PMA. The Ser^{1248} phosphorylation of PLC-γ1 was analyzed by immunoblotting with antibodies to PLC-γ1 phosphorylated on Ser^{1248} (anti-pSer^{1248}) (27). Expression of AHNAK did not affect Ser^{1248} phosphorylation by PMA (Fig. 6*A*). It has been established that PLC-γ1 is activated via tyrosine phosphorylation at tyrosines 771, 783, and 1254 in response to growth factor stimulation. Phe substitution of Tyr^{783} completely blocks the activation of PLC-γ by PDGF in NIH3T3 cells (28). Therefore, Tyr^{783} phosphorylation of PLC-γ1 is essential in the PTK-dependent activation of PLC-γ1. When we examined the effect of 4 CRUs on PKC activity in response to PMA, we noted that stimulation of cells expressing the 4 CRUs of AHNAK with PMA did not lead to phosphorylation at Tyr^{783} of PLC-γ1 (Fig. 6*B*). These results collectively indicate that the 4 CRUs of AHNAK stimulate PTK-independent activation of PLC-γ isozymes in response to PMA stimulation and further suggests that AHNAK-mediated PLC-γ activation by PMA occurs independent of the phosphorylation of Tyr^{783} or Ser^{1248} of PLC-γ1.

Bradykinin Shows a Biphasic Activation of PLC in NIH3T3 Cells Expressing 4 CRUs of AHNAK—We next examined the biological relevance of the proposed model of AHNAK-mediated PLC-γ activation in cells. We used BK to generate diacylglycerol in a PLC-γ-dependent manner to recruit the PKC-AHNAK-PLC-γ complex to the cell membrane. BK activates PLC-γ as well as PLC-β through transactivation of Src and the epidermal growth factor receptor (7, 29, 30). To prevent transactivation of PTK, specific pharmacological inhibitors such as PP-1 and AG1478 were used. We found that stimulation of NIH3T3 cells with BK induced G$_q$-dependent PLC-β activation resulting in a monophasic generation of inositol phosphate (IP$_T$) within 1 min in the presence of PP-1 and AG1478, whereas cells expressing 4 CRUs of AHNAK showed a biphasic mode (Fig. 7*A*). It appears that the initial generation of IP$_T$ is in response to PLC-β activation, but the latter is from PLC-γ activation. To prove that the secondary peak in the cells expressing 4 CRUs of AHNAK was generated by PLC-γ1 activation, we again utilized siRNA-mediated knockdown of PLC-γ1 as described in Fig. 3*A*. Silencing of PLC-γ1 in NIH3T3 cells expressing 4 CRUs of AHNAK resulted in disappearance of the secondary peak in response to BK, whereas control vector-transfected cells showed a biphasic mode of IP$_T$ generation (Fig. 7*B*). The result strongly supports that the latter peak in the IP$_T$ generation is from activation of the PLC-γ1 isozyme.

DISCUSSION

The AHNAK protein of ∼700 kDa can be functionally divided into three distinct regions: NH$_2$-terminal, central, and COOH-terminal regions. The central region is composed of a highly conserved 128-amino acid long motif repeated 36 times with the degree of amino acid identity between any two of these repeats being at least 80% (1–3). It has been suggested that the central repeated units in AHNAK form a β-strand and thin polyionic rod that provides the site for interacting with S100B, a calcium- and zinc-binding protein (5) and PLC-γ isozyme (9). In addition, the COOH-terminal region of AHNAK was shown to interact with L-type calcium channels in cardiomyocytes (4). Based on theses observations, AHNAK was proposed to be a molecular scaffold for intracellular calcium homeostasis in interaction with PLC, S100B, and calcium channels.

Although the AHNAK protein was originally identified as a nuclear protein, several lines of evidence suggest that the protein is localized in the plasma membrane (3, 31–33). Gentil *et al.* (31) reported that the main localization of AHNAK is at the plasma membrane in adult muscle cells and the lining of the epithelium. Hashimoto *et al.* (3) reported the PMA-induced translocation of whole AHNAK protein to plasma membrane in

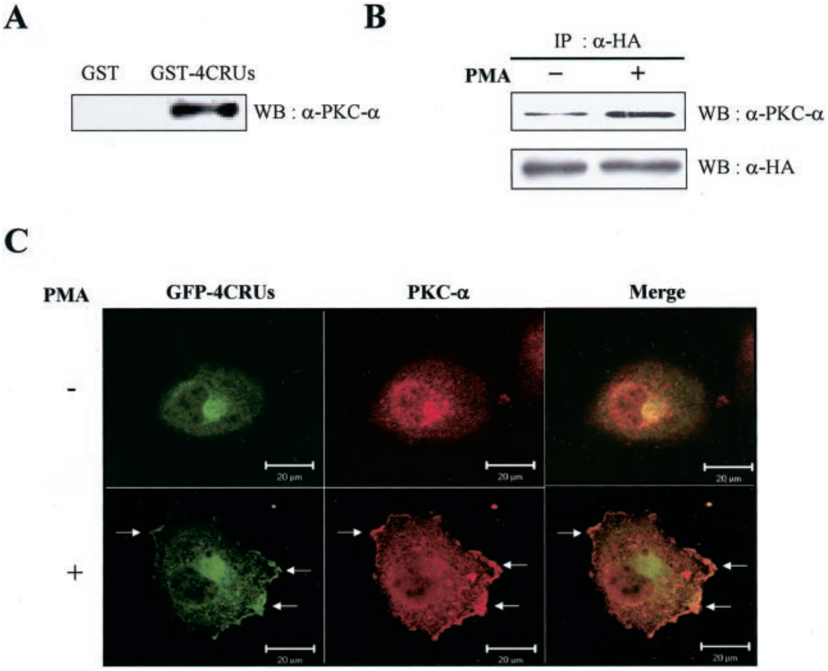

FIG. 5. **4 CRUs of AHNAK interact with PKC-α.** *A*, lysates from NIH3T3 cells were incubated with the GST-4CRU fusion protein or control GST protein, and GST-4CRU or GST protein precipitates were analyzed by SDS-PAGE and immunoblotting with anti-PKC-α. *B*, the cell lysates were immunoprecipitated with anti-HA and then analyzed by immunoblotting using anti-PKC-α (*upper panel*) or anti-HA (*lower panel*) antibodies. *C*, NIH3T3 cells expressing GFP-4CRU were unstimulated (*upper panel*) or stimulated with 100 nM PMA for 10 min (*lower panel*) and fixed and stained with PKC-α. TRITC-conjugated goat anti-mouse IgG was used as the secondary antibody for staining PKC-α. *WB*, Western blot.

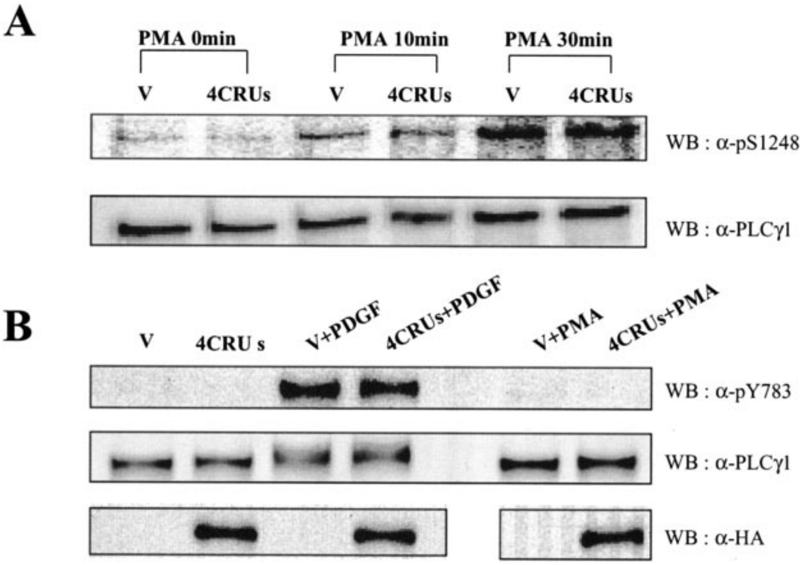

FIG. 6. **PMA-induced AHNAK-mediated PLC-γ1 activation is independent of the phosphorylation of Tyr^{783} or Ser^{1248} of PLC-γ1.** *A*, serum-starved NIH3T3 cells overexpressing pcDNA3-HA and pcDNA3-HA-4CRU were stimulated with 100 ng/ml PDGF or 100 nM PMA for 10 min. The cell lysates were immunoblotted with antibodies against pSer^{1248} of PLC-γ1 (*upper panel*) or PLC-γ1 (*lower panel*). *B*, cell lysates were immunoblotted with antibodies against pTyr^{783} of PLC-γ1 (*upper panel*), PLC-γ1 (*middle panel*), or HA (*lower panel*). *WB*, Western blot.

the immunofluorescence microscope with polyclonal antibodies to AHNAK. The result suggested an involvement of PKC in translocation of the AHNAK protein in keratinocytes (3). It has also been reported that high calcium influx and nerve growth factor induced the translocation of the AHNAK protein to the plasma membrane (32, 33). Although a series of reports suggested that the AHNAK protein localizes into the plasma membrane, the molecular mechanism for translocation of AHNAK is far from clear. Our results imply that the central repeated units are likely involved in the translocation mechanism of the AHNAK protein in response to PMA (Figs. 4 and 5).

We previously reported (8, 9) that both tau and AHNAK can activate the PLC-γ isozyme in the presence of arachidonic acid *in vitro*. Our data show that the repeated units of the AHNAK protein interacted and activated the PLC-γ isozyme in response to PMA in NIH3T3 cells (Figs. 2 and 3). CRUs of AHNAK stimulated IP$_T$ generation (Fig. 2, *B* and *D*) and mobilized the intracellular calcium (Fig. 2*E*), but the NH$_2$- and COOH-terminal regions had no such effect. Moreover, co-immunoprecipitation experiments and laser-based confocal microscopic analysis showed that 4 CRUs of AHNAK interacted with PLC-γ1 (Fig. 4). Jenkins *et al.* (34) suggested that the SH3 domain of the PLC-γ isozyme acts as the binding site for the proline-rich region of the tau protein (34). Although the proline-rich region in 4 CRUs of AHNAK repeats is not matched with the SH3-binding motif in other signaling proteins, it may serve as the binding site for the SH3 domain of PLC-γ.

It is well established that PKC isozymes are activated by phospholipids, diacylglycerol, and calcium resulting from receptor-mediated cell signaling (10, 11). The kinases are targeted to a specific cellular location through PKC isozyme-specific binding partners to function in cell growth, differentiation, and survival. Substrates that interact with protein kinase C, which are phosphorylated by PKC, are proposed as the molecular linker between PKC activation and cell adhesion and spreading (13, 35). Receptors for activated protein kinase C are well known as a binding partner of PKC and are colocalized to the perinucleus in cardiac myocytes (12, 36). AHNAK protein was previously found to be translocated to the plasma membrane in response to PMA treatment (1–3). Our results indicate

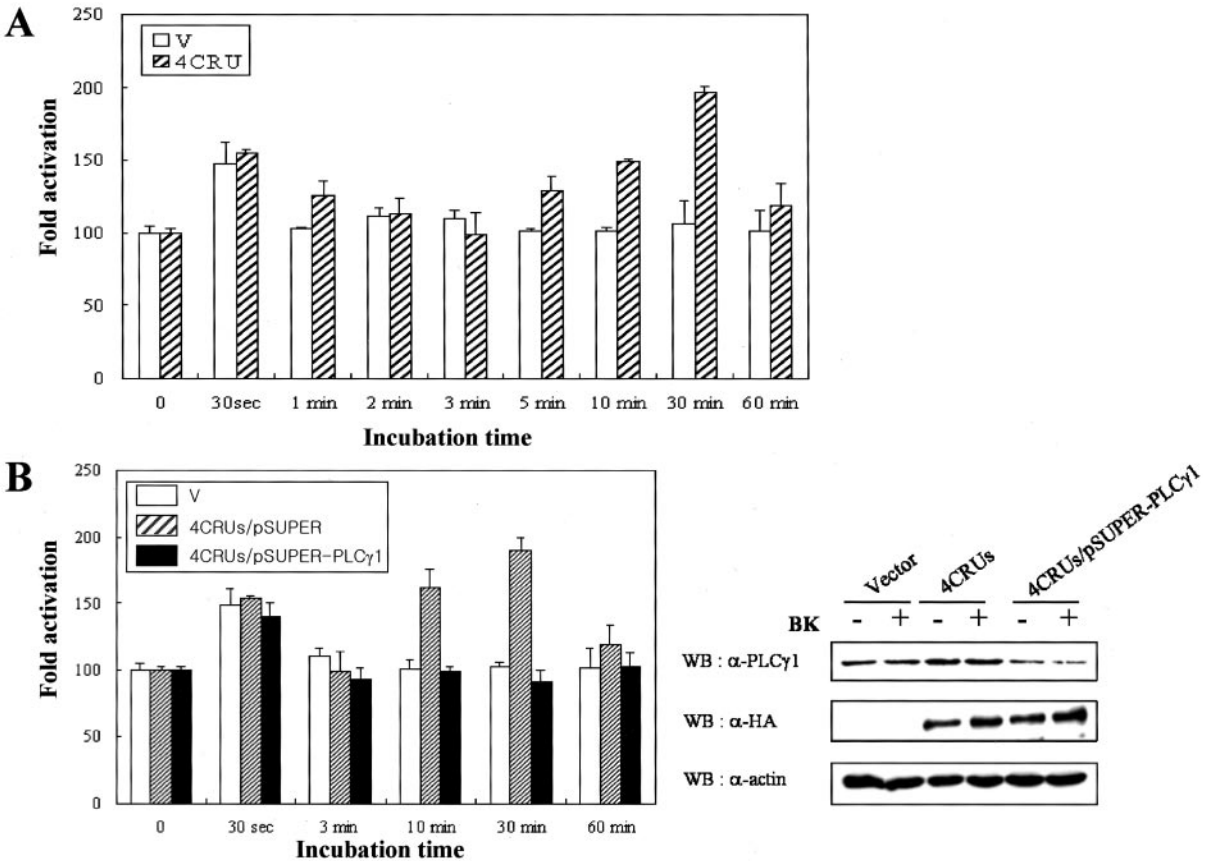

FIG. 7. **Bradykinin induces AHNAK-mediated PLC-γ1 activation.** *A*, NIH3T3 cells were transfected with pcDNA3-HA (*open bar*) or pcDNA3-HA-4CRU DNA (*shaded bar*). Cells were preincubated with PP1 and AG1478 and then treated with bradykinin for the indicated time periods. PLC activity was measured in NIH3T3 cells as described under "Experimental Procedures." Data are expressed as a percentage of the value for pcDNA3(HA)-transfected cells not treated with PMA and are average ± S.E. from three independent experiments. *B*, NIH3T3 cells were transfected with either pSUPER-PLC-γ1 encoding PLC-γ1-specific siRNA and HA or HA-4CRU. Control cells were transfected with pSUPER and pcDNA3 (HA). After culture for 36 h, the cells were labeled with inositol-free DMEM supplemented with *myo*-[2-^{3}H]inositol for 14 h. Cells were stimulated with PMA. IP_T generation as a PLC activity was measured as described under "Experimental Procedures." Data are mean ± S.E. of values from three independent experiments. Cell lysates were blotted with the indicated antibodies. *WB*, Western blot.

that 4 CRUs of AHNAK interact with PKC-α, which is the primary isozyme in NIH3T3 (Fig. 5). Consistently, a complex of repeats of the AHNAK with PKC is translocated to the plasma membrane (Fig. 5*C*). We found that repeated units of the AHNAK protein bind to classical PKCs, PKC-α (Fig. 5), as well as novel PKC and atypical PKCs, such as PKC-δ and PKC-ζ (data not shown). The classical PKC and novel PKCs contain a C1 domain that has conserved cystein and histidine residues that are responsible for the coordination of two Zn^{2+} ions (10, 11). The atypical PKC also contains a single zinc finger motif. Moreover, phosphatidylserine/diacylglycerol is no longer effective in enhancing PKC activity in the presence of a saturating concentration of AHNAK (data not shown). Taken together, these data suggest that the repeated units of the AHNAK protein likely interact with the PKC isozyme through the zinc binding motif in the C1 domain.

PKC in this complex had no effect either on tyrosine or serine phosphorylation of PLC-γ1. The question arises: what is the role of PKC in this model? A well established cellular function of PKC is MAPK activation. The 85-kDa $cPLA_2$ stimulates agonist-induced AA release. Membrane localization of $cPLA_2$ is regulated by the intracellular calcium level and agonist-dependent MAPK activation resulting in the release of AA (15, 17). According to our model, PKC in the complex would be translocated to the membrane and activate sequentially downstream from the cytosolic proteins such as $cPLA_2$ and MAPK.[2] We have also found that PMA-induced AA release, resulting from activation of $cPLA_2$ in NIH3T3 cells (Fig. 1). It has been reported that bradykinin stimulated the release of AA (37, 38). Both mechanisms for the generation of AA and activation of $cPLA_2$ activity and AHNAK protein would convey activation of the PLCγ isozyme.

We have also verified the biological relevance of the PKC-AHNAK-PLC-γ1 complex. NIH3T3 cells expressing 4 CRUs of AHNAK showed a biphasic generation of IP_T in response to BK. Moreover, the latter peak in IP_T generation results from activation of PLC-γ1 as shown by the PLC-γ1 depletion experiment (Fig. 7*B*). We, however, have found that the pattern of intracellular calcium mobilization in NIH3T3 cells expressing 4 CRUs of AHNAK in response to BK is similar to that of the parental cells (data not shown). These results indicate that the PKC-AHNAK-PLC-γ1 complex plays an important role in the generation of DAG rather than mobilization of intracellular calcium. The order of substrate preference for PLCγ1 is PI > PIP > PIP_2.[3] Activation of PLC-γ1 generates IP_3 as well as IP and IP_2. Moreover, BK-mediated PLCβ activation led to a shortage of PIP_2 in the plasma membrane. It is likely thus that IP and IP_2 are the major components in the latter peak of IP_T by BK stimulation in NIH3T3 cells expressing 4 CRUs of AHNAK.

AHNAK protein contains highly conserved 36 repeated units. Why does the AHNAK protein contain so many repeated

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[2] I. H. Lee and Y. S. Bae, unpublished results.

[3] Y. S. Bae and S. G. Rhee, unpublished observations.

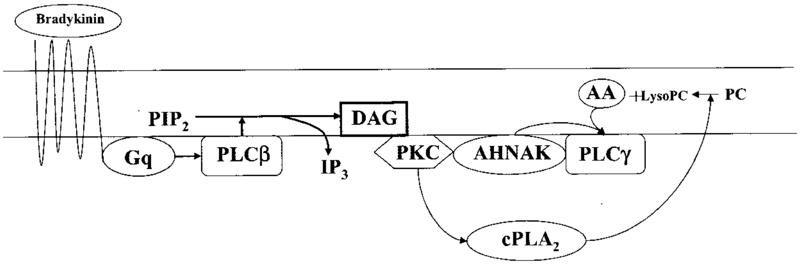

FIG. 8. **Putative model of the PLC-γ activation pathway through AHNAK and PKC.** Bradykinin-mediated PLC-γ isozyme activation results in activation of PKC. PLA$_2$ is sequentially stimulated by activation of PKC, leading the release of AA. A concerted action of central repeated units of AHNAK and AA induces PLC-γ1 activation.

units? A growing body of recent evidence indicates that many proteins with such repeated motifs serve as scaffolding molecules (39–43). For example, PDZ domains are protein-protein recognition motifs involved in specific cell signaling cascades (41). The *Drosophila* protein INAD contains five PDZ domains that provide scaffolding for signaling molecules in the G-protein-coupled phototransduction cascade. The protein simultaneously interacts with PLC, eye PKC, and the transient receptor potential Ca^{2+} channel forming multiple complexes that convey a rapid and efficient signal transduction (41). PR65/A contains 15 tandemly repeated HEAT motifs that serve as a binding site for protein phosphatase 2A, SV40 small T antigen, polyoma virus small and middle T antigen (42, 43). Mutations in the HEAT motif of PR65A are closely associated with lung and colon cancer development (42). This report suggests that destabilization of protein folding by the mutations interferes with protein-protein interactions resulting in tumorigenesis. Recently, we have found that the CRU of AHNAK interacts not only with PLC-γ and PKC but also with sentrin and Smad 1 protein, which are key mediators of sumoylation and transforming growth factor-β signaling, respectively.[2] How molecular mechanisms involving PLC-γ, PKC, sentrin, and Smad 1 protein are integrated into mediating a physiological signal remains to be elucidated.

In summary, we present here a putative model suggesting that 4 CRUs of AHNAK interact with PKC and PLC-γ1 simultaneously (Fig. 8). PKC is activated by diacylglycerol, which is a product of PLC-β activation, and by interaction with 4 CRUs of AHNAK. Activation of PKC stimulates the release of AA resulting from cPLA2 activation. A concerted action of 4 CRUs of AHNAK and AA induces PLC-γ1 activation. The model thus suggests a novel PLC-γ1 activation pathway that includes the combined activity of AHNAK and PKC.

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AHNAK-mediated Activation of Phospholipase C-γ1 through Protein Kinase C

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