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-γ 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 (IPT) and mobilization of the intracellular calcium. Phorbol 12-myristate 13-acetate-dependent generation of IPT was completely blocked in NIH3T3 cells depleted of PLC-γ by RNA interference. Furthermore, bradykinin, which normally stimulated the PLC-β isozyme resulting in the generation of a monophasic IPT within 30 s in NIH3T3 cells, led to a biphasic pattern for generation of IPT in NIH3T3 cells expressing 4 CRUs of AHNAK. The second peak of 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 NH₂-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 Ca²⁺ 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 (6) 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 heptasquence motif, (D/E)₁₆(κ/θ)₁₀(G/P)₁₆, where φ and Ω represent hydrophobic and hydrophilic amino acid residues, respectively. Shitvenman 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 (PIP₂) to produce inositol (1,4,5)-trisphosphate (IP₃) and 1,2-diacylglycerol (DAG). To date, a total of 11 different isoforms 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 isoforms. 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)-trisphosphate, 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).

* The abbreviations used are: PKB, protein kinase B; PLC, phospholipase C; PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₃, inositol (1,4,5)-trisphosphate; DAG, 1,2-diacylglycerol; PTK, protein-tyrosine kinase; AA, arachidonic acid; MAPK, mitogen-activated protein kinase; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; FMA, 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; IPT, total inositol phosphate; PBS, phosphate-buffered saline; [Ca²⁺]i, intracellular Ca²⁺; SH3, Src homology domain 3.

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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. δ, ε, θ, and η can also be activated by DAG and phorbol esters but are Ca\(^{2+}\)-independent. The atypical PKCs, which include PKCζ and PKCs, 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 phagocytosis 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 AHNAK 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\(_6\) MicroBeads were obtained from Miltenyi Biotech, 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 5525–5643) domain was based on a previous 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 SpeI 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, pcDNA3-HA-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 × 100 and then normalized to the value of unstimulated controls (18, 19).

**Measurement of Total Inositol Phosphate ([IP\(_3\)]\(_g\)) in Cells**—NIH3T3 cells were labeled with inositol-free DMEM supplemented with myo-[2-\(^3\)H]inositol (1 μCi/ml, 25 μCi/nmol) (DuPont Biotechnology Systems) and pEGFP-N1 and pDsRed-C1 (Clontech) were subsequently incubated in DMEM containing 20 μM LiCl for 30 min and then 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 ml 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\(_3\), was measured using a liquid scintillation counter.

**Magnetic Enrichment of NIH3T3 Cells Expressing AHNAK—NIH3T3 cells were co-transfected pMACS K\(_6\) 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 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 ended by adding 100% 10% fetal bovine serum, and then incubated with 80 μl of MACSelect K\(_6\) 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\(_6\) 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-[2-\(^3\)H]inositol (1.5 μCi/ml, 25 μCi/nmol) (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–CRUs. Transfected NIH3T3 cells were starved for 12 h, incubated with 2 mM 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. The peak emission of 100 μM 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-γ**—Specific sequences of 19-nucleotide sequence (siRNA-ACCGAGCAGGACCA GAAACGG) and SMARTools (17) were used for synthesis of a siRNA. sSUPER vector for siRNA was purchased from Oligogene (22). The phosphorylated oligonucleotides were annealed and cloned into the sSUPER 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 with 1 μM PMA for 5 min in the absence or presence of 10 μM AG1478 or for the indicated times in the absence of bradykinin, and then analyzed by measurement of total inositol phosphates. The depletion of endogenous PLC-γ 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-aminoethylbenzenesulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) and lysed by sonication. After centrifugation at 15,000 × 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 analysis.

Immunoprecipitation and Immunoblotting—Lysates (1–2 × 106 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, 50 ng/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 cPLA2 and then release AA. To verify the cellular function of AHNAK, we transfected NIH3T3 cells with pSUPER-PLC-1 gene (22). The cells transfected with the siRNA vector expressing 4 CRUs of AHNAK, NIH3T3 cells were transfected with 4 CRUs of AHNAK in NIH3T3 cells (Fig. 3A). NIH3T3 cells transfected with the empty pSUPER vector (Fig. 3A). NIH3T3 cells transfected with the pSUPER-PLC-γ vector failed to generate IP3 in response to PMA, whereas the cells transfected with pSUPER alone exhibited a marked increase of IP3 generation in response to PMA.

To prove the selectivity for PLC-γ in an AA-depleted in vitro setting, we transfected NIH3T3 cells with RFP-4CRU, and measured the production of IP3 as an indicator of PLC-γ activation, we transfected NIH3T3 cells 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).
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 IP₃ generated was measured as described under...
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-\(\gamma\)1, PLC-\(\gamma\)2, PLC-\(\gamma\)3, and PLC-\(\gamma\)4, respectively. PLC-\(\gamma\)3 is the predominantly expressed PLC-\(\gamma\) isozyme in NIH3T3 cells.

The 4 CRUs of the AHNAK protein interacted with PLC-\(\gamma\)3 in a PMA-dependent manner, whereas other isozymes failed to interact with AHNAK (Fig. 4A). To investigate whether platelet-derived growth factor (PDGF) stimulates the interaction of 4 CRUs of AHNAK with PLC-\(\gamma\)3, 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-\(\gamma\)3. The result indicates that 4 CRUs of the AHNAK protein failed to interact with PLC-\(\gamma\)3 in response to PDGF suggesting that AHNAK mediates PTK-independent PLC-\(\gamma\) activation (Fig. 4B).

Moreover, we have observed colocalization of PLC-\(\gamma\) with AHNAK in response to PMA. Endogenous PLC-\(\gamma\) and GFP-4CRU were located in the cytoplasm and perinuclear regions in resting cells, whereas GFP-4CRU was co-localized with PLC-\(\gamma\) in plasma membrane (arrow) and the perinuclear region in response to PMA stimulation (Fig. 4C). Asterisks indicate NIH3T3 cells untransfected with 4 CRUs of AHNAK. Plasma membrane localization of PLC-\(\gamma\) 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-PLC-\(\gamma\) complex in the plasma membrane.

Central Repeated Units of AHNAK Protein Interact with PKC Isozyme—Because NIH3T3 cells appear to mainly express the PKC-\(\alpha\) isozyme (25), we employed co-immunoprecipitation experiments to assess interaction between AHNAK and PKC-\(\alpha\).

**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 KK with vector 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-[\(3^3\)H]inositol for 14 h and stimulated with PMA. IP production by PLC-\(\gamma\) 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-\(\gamma\) were coexpressed in PLC-\(\gamma\)-null mouse embryonic fibroblasts (TV1-null cells). After culture for 36 h, cells were stimulated with PMA. IP production as a PLC-\(\gamma\) 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.
we have observed that PKC in the absence of PMA, whereas GFP-4CRUs (Fig. 5A) in plasma membrane upon PMA stimulation (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. 5C).

 Several lines of evidence suggest that PMA induces phosphorylation of Ser1248 in PLC-γ1 (26). For example, PKC desensitized the PLC-γ1 activity upon OKT-3 activation via Ser1248 phosphorylation in Jurkat T cells (26). To elucidate the role of PKC in the complex, we investigated the effect of 4 CRUs on Ser1248 phosphorylation of PLC-γ1 in response to PMA. The Ser1248 phosphorylation of PLC-γ1 was analyzed by immunoblotting with antibodies to PLC-γ1 phosphorylated on Ser1248 (anti-pSer1248) (27). Expression of AHNAK did not affect Ser1248 phosphorylation by PMA (Fig. 6A). It has been established that PLC-γ1 is activated via tyrosine phosphorylation at tyrosines 771, 783, and 1254 in response to growth factor stimulation. The substitution of Tyr783 in the ternary complex of PKC-AHNAK-PLC-γ1 completely blocks the activation of PLC-γ1 by PDGF in NIH3T3 cells (28). Therefore, Tyr783 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 Tyr783 of PLC-γ1 (Fig. 6B). These results collectively indicate that the 4 CRUs of AHNAK stimulate PTK-independent activation of PLC-γ isoforms in response to PMA stimulation and further suggest that AHNAK-mediated PLC-γ activation by PMA occurs independent of the phosphorylation of Tyr783 or Ser1248 of PLC-γ1.

**DISCUSSION**

The AHNAK protein of ~700 kDa can be functionally divided into three distinct regions: NH2-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 polycyclic rod that provides the site for interacting with S100B, a calcium- and zinc-binding protein (5) and PLC-γ isoforms (9). In addition, the COOH-terminal region of AHNAK was shown to interact with L-type calcium channels in cardiomyocytes (4). Based on these 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...
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$_2$ generation (Fig. 2, B and D) and mobilized the intracellular calcium (Fig. 2E), 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-γ1 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-γ1.

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...
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 protein binds to classical PKCs, PKC-α (Fig. 5), as well as novel PKCs 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 Zn2⁺ ions (10, 11). The atypical PKC also contains a single zinc finger motif. Moreover, phosphatidylinositol/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 cPLA₂ stimulates agonist-induced AA release. Membrane localization of cPLA₂ 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 cPLA₂ and MAPK.² We have also found that PMA-induced AA release, resulting from activation of cPLA₂ 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 cPLA₂ activity and AHNAK protein would convey activation of the PLC-γ isozyme.

We have also verified the biological relevance of the PKC-AHNAK-PLC-γ complex. NIH3T3 cells expressing 4 CRUs of AHNAK showed a biphasic generation of IP₃ in response to BK. Moreover, the latter peak in IP₃ generation results from activation of PLC-γ1 as shown by the PLC-γ1 depletion experiment (Fig. 7B). 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-γ complex plays an important role in the generation of DAG rather than mobilization of intracellular calcium. The order of substrate preference for PLC-γ1 is PIP₂ > PIP > PIP₃.³ Activation of PLC-γ1 generates IP₃ as well as IP and IP₂. Moreover, BK-mediated PLC-γ activation led to a shortage of PIP₂ in the plasma membrane. It is likely thus that IP and IP₂ are the major components in the latter peak of IP₃ 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 units?
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 Ca2+ 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 PR65/A 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. 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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