

# Requirements for Pseudosubstrate Arginine Residues during Autoinhibition and Phosphatidylinositol 3,4,5-(PO<sub>4</sub>)<sub>3</sub>-dependent Activation of Atypical PKC\*

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Robert A. Ivey<sup>‡</sup>, Mini P. Sajan<sup>‡§</sup>, and Robert V. Farese<sup>‡§1</sup>

From the <sup>‡</sup>Medical and Research Services, James A. Haley Veterans Medical Center, Tampa, Florida 33612 and the <sup>§</sup>Division of Endocrinology and Metabolism, Department of Internal Medicine, University of South Florida College of Medicine, Tampa, Florida 33612

**Background:** The mechanisms whereby atypical PKCs (aPKCs) are silenced and activated by phosphatidylinositol 3,4,5-(PO<sub>4</sub>)<sub>3</sub> are uncertain.

**Results:** Mutagenesis of arginine residues in the pseudosubstrate of aPKCs caused constitutive activation that was inhibited by exogenous pseudosubstrate. Pseudosubstrate-dependent inhibition was reversed by phosphatidylinositol 3,4,5-(PO<sub>4</sub>)<sub>3</sub>.

**Conclusion:** Pseudosubstrate arginine residues are required for autoinhibition and are targeted by phosphatidylinositol 3,4,5-(PO<sub>4</sub>)<sub>3</sub> during aPKC activation.

**Significance:** Pseudosubstrate arginine residues are key regulators of aPKC.

Atypical PKC (aPKC) isoforms are activated by the phosphatidylinositol 3-kinase product phosphatidylinositol 3,4,5-(PO<sub>4</sub>)<sub>3</sub> (PIP<sub>3</sub>). How PIP<sub>3</sub> activates aPKC is unknown. Although Akt activation involves PIP<sub>3</sub> binding to basic residues in the Akt pleckstrin homology domain, aPKCs lack this domain. Here we examined the role of basic arginine residues common to aPKC pseudosubstrate sequences. Replacement of all five (or certain) arginine residues in the pseudosubstrate sequence of PKC- $\iota$  by site-directed mutagenesis led to constitutive activation and unresponsiveness to PIP<sub>3</sub> *in vitro* or insulin *in vivo*. However, with the addition of the exogenous arginine-containing pseudosubstrate tridecapeptide to inhibit this constitutively active PKC- $\iota$ , PIP<sub>3</sub>-activating effects were restored. A similar restoration of responsiveness to PIP<sub>3</sub> was seen when exogenous pseudosubstrate was used to inhibit mouse liver PKC- $\lambda/\zeta$  maximally activated by insulin or ceramide and a truncated, constitutively active PKC- $\zeta$  mutant lacking all regulatory domain elements and containing “activating” glutamate residues at loop and autophosphorylation sites ( $\Delta$ 1–247/T410E/T560E-PKC- $\zeta$ ). NMR studies suggest that PIP<sub>3</sub> binds directly to the pseudosubstrate. The ability of PIP<sub>3</sub> to counteract the inhibitory effects of the exogenous pseudosubstrate suggests that basic residues in the pseudosubstrate sequence are required for maintaining aPKCs in an inactive state and are targeted by PIP<sub>3</sub> for displacement from the substrate-binding site during kinase activation.

The atypical PKC (aPKC)<sup>2</sup> isoforms PKC- $\zeta$ , PKC- $\lambda$ , and primate-specific PKC- $\iota$  serve as critically important kinases in multiple cellular processes. Indeed, total knockout of PKC- $\lambda/\iota$  is embryonic lethal in mice, and alterations in aPKC in humans are important determinants in diverse disorders, including various cancers, where aPKC activity is increased frequently (1), and obesity and type 2 diabetes mellitus, where aPKC activity is diminished in muscle (2–4) and adipocytes (4), where aPKC and Akt are required for insulin-stimulated glucose transport, but inordinately increased in the liver (5), where aPKC and Akt are required for insulin-stimulated lipid synthesis. Despite their importance, little is known about the molecular mechanisms that are operative in aPKC activation.

Like Akt, aPKCs are activated by PI3K during actions of insulin (6) and other growth factors, *e.g.* insulin-like growth factor 1 (7). The activation of both aPKC and Akt by PI3K is effected by generation of phosphatidylinositol-3,4,5-(PO<sub>4</sub>)<sub>3</sub> (PIP<sub>3</sub>) from phosphatidylinositol-4,5-(PO<sub>4</sub>)<sub>2</sub> (PIP<sub>2</sub>) in the plasma membrane and other cellular compartments. Akt activation is thought to be initiated by an interaction of the acidic D3-phosphoinositide group of PIP<sub>3</sub>, with basic arginine and lysine residues in the pleckstrin homology (PH) domains contained in both Akt (8) and phosphoinositide-dependent protein kinase 1 (PDK1) (9). This colocalization allows PDK1 to phosphorylate threonine 308 in the Akt activation loop, which, in turn, facilitates phosphorylation of serine 473 in the C-terminal hydrophobic motif of Akt by “PDK2,” now identified as the mammalian target of rapamycin 2 (mTORC2) (10). Whether PIP<sub>3</sub> increases the activity of PDK1 or mTORC2 is debatable (*e.g.* see Ref. 3). In any case, unlike Akt, aPKCs do not have a PH domain, and how PIP<sub>3</sub> either localizes or activates aPKC at the molecular level is presently obscure. On the other hand, like Akt, the D3-PO<sub>4</sub> group appears to mediate the activation of aPKC by

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<sup>1</sup> To whom correspondence should be addressed: Research Service, James A. Haley Veterans Medical Center, 13000 Bruce B. Downs Blvd., Tampa, FL 33612. Tel.: 813-972-2000 (ext. 4414) or 813-766-5657 (cell phone); Fax: 813-972-7623; E-mail: rfarese@health.usf.edu.

<sup>2</sup> The abbreviations used are: aPKC, atypical PKC; PIP<sub>3</sub>, phosphatidylinositol 3,4,5-(PO<sub>4</sub>)<sub>3</sub>; PIP<sub>2</sub>, phosphatidylinositol-4,5-(PO<sub>4</sub>)<sub>2</sub>; PI, phosphatidylinositol; PH, pleckstrin homology; DAG, diacylglycerol; Ni-NTA, nickel-nitrilotriacetic acid.

## Regulation of aPKC Activity

PIP<sub>3</sub> because PI-3,4-(PO<sub>4</sub>)<sub>2</sub>, but not PI-4,5-(PO<sub>4</sub>)<sub>2</sub>, increases aPKC phosphorylation (11).

As with Akt, as well as with conventional PKCs ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) and novel PKCs ( $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$ ), aPKC activation requires phosphorylation of threonine residues in activation loops, *viz.* threonine 412 in PKC- $\iota$ , threonine 411 in PKC- $\lambda$ , and threonine 410 in PKC- $\zeta$  by PDK1, and auto(trans)phosphorylation sites, *viz.* threonine 564 in PKC- $\iota$ , threonine 563 in PKC- $\lambda$ , and threonine 560 in PKC- $\zeta$  in the turn regions of their catalytic domains (11, 12). In addition to required phosphorylations of loop and auto(trans)phosphorylation sites, PIP<sub>3</sub> has been postulated to provoke allosteric effects during aPKC activation (12).

Like conventional and novel PKCs, aPKCs exist basally in a folded inactive state in which residues in the pseudosubstrate region of the regulatory domain bind to residues in the substrate-binding region of the catalytic domain. Therefore, it may be hypothesized that, in aPKCs, basic arginine residues in the pseudosubstrate sequence bind to acidic residues in the substrate-binding region and that disruption of this binding by an acidic ligand, such as PIP<sub>3</sub>, leads to molecular unfolding and exposure of the substrate-binding site not only to extrinsic substrates but also to the intrinsic auto- or trans-phosphorylation site. In this regard, note that, with conventional and novel PKCs, this unfolding and subsequent activation is effected by Ca<sup>2+</sup>, which binds to a site in the C2 region of the regulatory domain that is uniquely present in conventional PKCs, and diacylglycerol (DAG), which binds to sites in the C1 region of the regulatory domain that are present in both conventional and novel PKCs. Interestingly, it is thought that one potential DAG-binding site is also present in the C1 region of aPKCs but is functionally blocked by a set of four basic arginine residues that surrounds the opening to this pocketed activation site (13). Accordingly, these arginine residues in the "DAG pocket ring" could reasonably be a point of attack for PIP<sub>3</sub>.

In the case of aPKCs, it is currently uncertain how insulin or other PI3K activators use PIP<sub>3</sub> to either localize aPKCs to the plasma membrane in juxtaposition with PDK1 or induce molecular unfolding and subsequent activation by auto(trans)phosphorylation. However, given the fact that the aPKC pseudosubstrate sequence, like the aPKC consensus substrate recognition sequence, contains basic arginine residues that flank a short sequence that, in substrates, contains phosphorylatable threonine or serine residues, we examined the importance of the five basic arginine residues that are common to pseudosubstrate sequences of all aPKCs for their ability to maintain aPKC in an inactive state and serve as a target used by PIP<sub>3</sub> to provoke a dissociation of the pseudosubstrate from the substrate-binding site and, thereby, promote kinase activation.

## EXPERIMENTAL PROCEDURES

**Cell Culture and Incubation Conditions**—3T3/L1 adipocytes were differentiated, cultured, and transfected with plasmids or infected with adenoviruses and incubated for 48–72 h to allow time for expression, as described previously (14). At the time of the experiment, the medium was removed, and cells were preincubated for 3 h in Krebs-Ringer phosphate buffer containing 5 mM D-glucose and then incubated for 30 min in glucose-free Krebs-Ringer phosphate buffer  $\pm$  100 nM insulin (Sigma). For

studies of glucose transport, the cells were subsequently incubated for another 5 min with 0.05 mM D-glucose and a trace amount of [<sup>3</sup>H]2-deoxyglucose, as described previously (14). After incubation, the cells were washed three times with cold medium, released from incubation plates with 0.1% sodium dodecyl sulfate solution, and sonicated in buffer as described below. Portions of cell lysates were placed into Laemmli buffer for Western blot analysis, used for measurement of protein levels and [<sup>3</sup>H]2-deoxyglucose uptake, used for immunoprecipitation of epitope-tagged or total aPKCs, or used for harvesting of His<sub>6</sub>-tagged aPKCs by adsorption with Ni-NTA-coated beads (EMD Millipore, Billerica, MA).

**Mouse Studies**—In some experiments, we used aPKCs immunoprecipitated from livers of C57Bl/6-SV/129 mice that were treated intraperitoneally with maximally effective insulin (1 unit/kg body weight) or vehicle 15 min before killing, as described previously (15, 16).

**Cell Lysate Preparations**—As described previously (14–16), adipocytes were sonicated, and mouse liver was homogenized in ice-cold buffer containing 0.25 mM sucrose, 20 mM Tris-HCl (pH 7.5), 2 mM EGTA, 2 mM EDTA, 1 mM PMSF, 20  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 2 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM NaF, and 1  $\mu$ M microcystin; supplemented with 1% Triton X-100, 0.6% Nonidet, and 150 mM NaCl; and cleared by low-speed centrifugation.

**aPKC Activation**—aPKC activity was measured essentially as described previously (6, 17) using His<sub>6</sub>-tagged aPKCs isolated from 3T3/L1 adipocyte lysates by adsorption onto Ni-NTA beads and eluted with assay buffer containing 100 mM EDTA, using HA-tagged aPKC isolated by immunoprecipitation with rabbit polyclonal anti-HA-purified I $\gamma$ G (Santa Cruz Biotechnology, Santa Cruz, CA, or using total aPKC isolated from lysates of 3T3/L1 adipocytes or mouse liver by immunoprecipitation with polyclonal antiserum (Santa Cruz Biotechnology) that recognizes the C termini of PKC- $\lambda/\iota$  and PKC  $\zeta$ . Immunoprecipitated aPKC was collected on Sepharose-AG beads. Isolated aPKC was incubated for 8 min at 30 °C in 100  $\mu$ l of buffer containing 50 mM Tris-HCl (pH7.5), 100  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 100  $\mu$ M Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM NaF, 100  $\mu$ M PMSF, 4  $\mu$ g of phosphatidylserine (Sigma), 50  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (NEN/Life Science Products, Waltham, MA), 5 mM MgCl<sub>2</sub> and, as substrate, 40  $\mu$ M serine analog of the PKC- $\epsilon$  pseudosubstrate (Enzo Life Sciences, Farmingdale, NY). After incubation, <sup>32</sup>P-labeled substrate was trapped on P-81 filter paper and counted in a liquid scintillation counter. Note that phosphatidylserine serves as a cofactor by providing a phospholipid surface that binds and optimizes PKC activity during activation by diacylglycerol and other lipids (18–21); that, as shown in Fig. 1, both the phospholipid cofactor phosphatidylserine and the aPKC substrate are routinely present at optimal concentrations in the assay; and that insulin, via endogenous PIP<sub>3</sub>, provokes increases in activity that are additive to the dose-dependent effects of phosphatidylserine. In some cases, aPKC activation was also assessed by immunoblot analysis of phospho-Thr-563/560-PKC- $\zeta/\lambda$ .

**Western Blot Analyses**—As described previously (2–5), harvested plasmids or cell lysates were immunoblotted for PKC- $\zeta/\lambda$  using a rabbit polyclonal antiserum (Santa Cruz Biotechnology) that recognizes the C termini of all aPKCs;  $\lambda$ ,  $\iota$ , and  $\zeta$ ; rabbit poly-

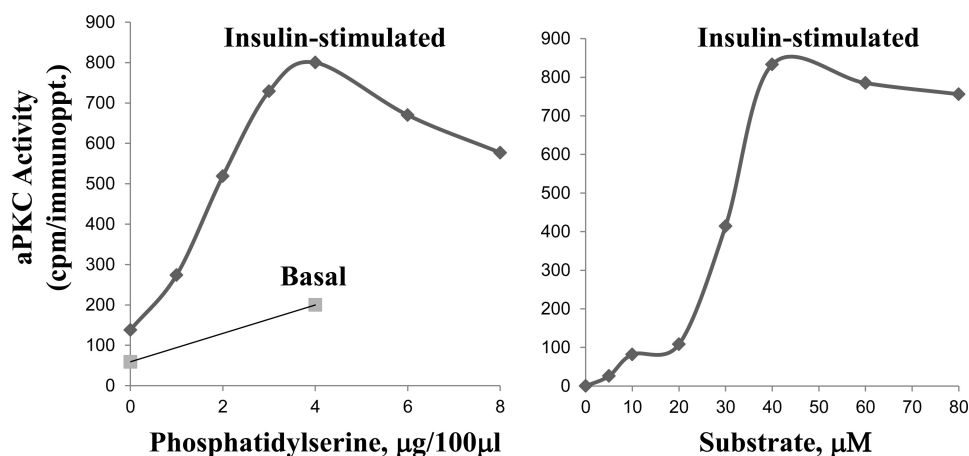


FIGURE 1. Dose-dependent requirements for phosphatidylserine (left panel) and substrate (right panel) during aPKC assay. Where indicated, mice were injected intraperitoneally with a maximally effective dose of insulin (1 unit/kg body weight) or saline vehicle 15 min before killing. aPKC was immunoprecipitated from liver lysates and assayed with increasing amounts of phosphatidylserine and a preferred aPKC substrate, *i.e.* the serine analog of the PKC- $\epsilon$  pseudosubstrate, along with other standard components of the aPKC assay (see "Experimental Procedures"). Data are mean of 2–4 replicates.

clonal anti-phospho-Thr-563/560-PKC- $\lambda/\zeta$  (Life Technology, Grand Island, NY); and rabbit polyclonal anti-phospho-Thr-411/410-PKC- $\lambda/\zeta$  (Cell Signaling Technology, Danvers, MA).

**Site-directed Mutagenesis and Preparation of Plasmids and Adenoviruses**—HA-tagged, constitutively active, truncated mutant HA- $\Delta$ 1–247/T410E/T560E-PKC- $\zeta$ , which lacks all regulatory domain elements and contains acidic glutamate residues at loop and autophosphorylation sites, was generated and cloned into the pCDNA3 plasmid as described previously (12).

For the generation of arginine-to-alanine mutations in the pseudosubstrate sequence and in the ring of arginine residues surrounding a pocket containing a potential DAG-binding site in the C1 region of PKC- $\iota$  (13), a clone of human PKC- $\iota$  was generated from a cDNA library prepared from human brain tissue using standard PCR techniques. The following primers were used to generate a PCR product suitable for direct recombination into the Gateway pDONR entry vector via BP Clonase II (Invitrogen): 5', GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGCATCATCATCATCATCATCATGCCCCGACCCAGAGGGACAGCAGCACC; 3', GGGGACCACTTTGTACAAGAAAGCTGGGTCTACTTCTCGAACTGGGGGTGGGACCTTCTCCGACACATTTCTTGACAGACATCAAAGAGG. To minimize extraneous amino acids in the expressed protein, Shine-Dalgarno and Kozak translation initiation sequences and a start codon were included, along with coding for a His<sub>6</sub> tag in the 5' primer. In addition, the 3' primer contained coding for a StrepII tag as well as a stop codon. PCR conditions included using 100 ng of cDNA for 35 cycles with denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 68 °C for 2 min.

Mutations of the human PKC- $\iota$  clone in the pDONR vector were generated using standard PCR techniques as follows: R147A/R150A/R151A/R160A (*i.e.* four arginine residues surrounding a putative DAG activation site) mutant, CCACACTTCCAAAGCCAAGGCTTTCAACGCGGCTGCTCACTGTGCCATCTGCACAGACGCAATATGGGACTTGGACG (5') and CGTCCAAGTCCCATATTGCGTCTGTGCAGATGGCACAGTGAGCAGCCGCGTTGAAAGCCTTGCTTGAAAGTGTGG (3'); R126A (*i.e.* a single arginine residue in

the pseudosubstrate sequence) mutant, GATAAATCCATCT-ACGCTAGAGGTGCACGCCGC (5') and GCGGCGTGCACCTCTAGCGTAGATGGATTTATCTTCTCC (3'); R131A (*i.e.* a single arginine residue in the pseudosubstrate sequence) mutant, CGTAGAGGTGCACGCGCCTGGAGAAAGCTTTATTGTGC (5') and ATAAAGCTTTCTCCAGGCGCGTGCACCTCTACG (3'); and R126A/R127A/R130A/R131A/R133A (all five arginine residues in the pseudosubstrate sequence) mutant, TAAATCCATCTACGCTGCAGGTGCAGCCGCC-TGGGCAAAGCTTTATTGTGCC (5') and GGCACAATAAGCTTTCCCAGGCGGCTGCACCTGCAGCGTAGATGATTTATCTTCTCC (3'). PCR conditions included using 1 ng of vector for 40 cycles with denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 68 °C for 10 min. All primers for cloning and mutating the PKC- $\iota$  gene were supplied by Eurofins MWG Operon (Huntsville, AL).

For direct, plasmid-based expression in mammalian cell culture, wild-type and mutant versions of PKC- $\iota$  were transferred to the Gateway vector pcDNA3.2 using LR Clonase II (Invitrogen). To generate recombinant adenoviruses for expression of the PKC- $\iota$  clones, wild-type and mutant versions were transferred to the pAD vector using LR Clonase II (Invitrogen). Standard transfection and viral amplification techniques were used for large scale expression of each clone.

**NMR Studies of PIP<sub>3</sub> Binding to the aPKC Pseudosubstrate**—NMR spectra were collected at 25 °C on a Varian VNMRS 600 MHz spectrometer equipped with a triple resonance pulse field *z* axis gradient cold probe. Two-dimensional 1H-1H TOCSY spectra had widths of 12 ppm in both dimensions with 2048 (t<sub>2</sub>) × 120 (t<sub>1</sub>) complex data points. NMR spectra were processed and analyzed with VnmrJ 3.2A (Varian). Samples (pseudosubstrate peptides (Genscript, Piscataway, NJ) and PIP<sub>3</sub> (Matreya)) were prepared in phosphate-buffered saline (pH 7.4) in 90% H<sub>2</sub>O, 10% D<sub>2</sub>O. NMR studies were performed by the Florida Center of Excellence for Drug Discovery at the University of South Florida.

**Statistical Methods**—Data are expressed as mean ± S.E. Statistical differences between two and three or more groups were determined by Student's *t* test and analysis of variance (Sigma Stat statistical software), respectively.

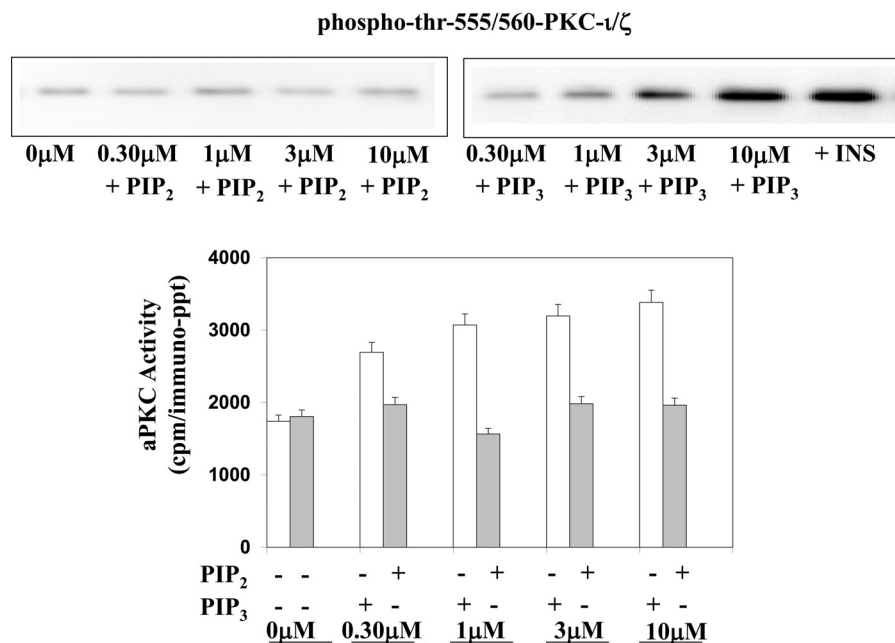


FIGURE 2. **Effects of PIP<sub>3</sub> and PIP<sub>2</sub> on activity and phosphorylation of aPKC.** aPKC was immunoprecipitated from lysates of livers harvested from basal (*i.e.* not insulin-stimulated) mice, and assayed in the presence of increasing concentrations of PIP<sub>3</sub> (*white columns*) or PIP<sub>2</sub> (*black columns*). Data are mean  $\pm$  S.E. of triplicate determinations. Also shown are representative immunoblots of alterations in autophosphorylation (phospho-thr-563/560-PKC- $\lambda/\zeta$ ).

## RESULTS

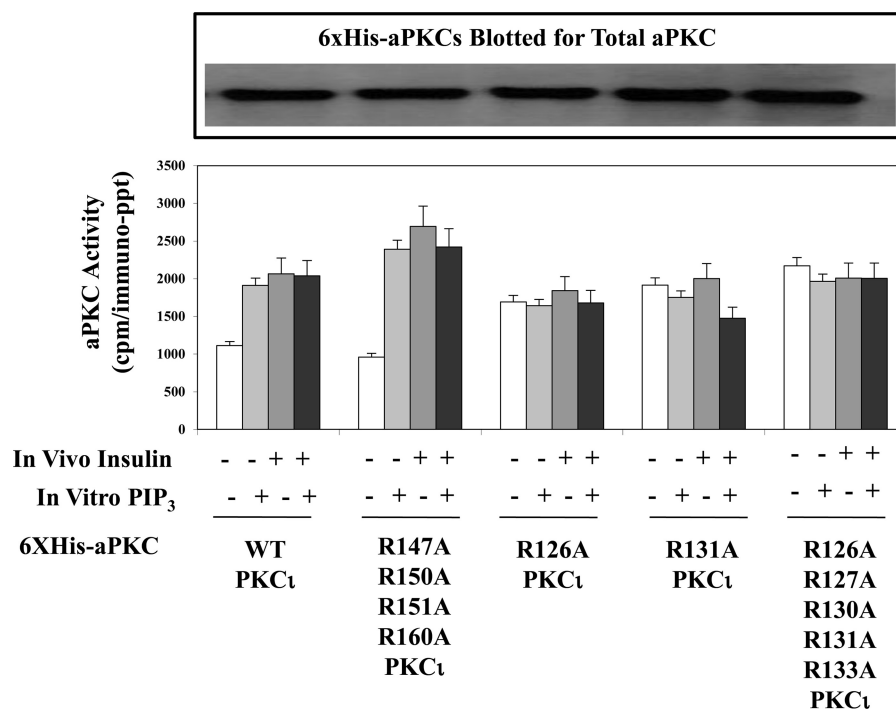
**Activation of aPKC by PIP<sub>3</sub> versus PIP<sub>2</sub>**—In keeping with previous findings of increased overall <sup>32</sup>P-PO<sub>4</sub> labeling of aPKC during incubation of immunoprecipitated aPKC with PIP<sub>3</sub>, but not PIP<sub>2</sub> (11), we found that PIP<sub>3</sub>, but not PIP<sub>2</sub>, increased both enzyme activity and autophosphorylation of aPKC immunoprecipitated from mouse liver (Fig. 2). These findings show that the D3-PO<sub>4</sub> group of PIP<sub>3</sub> is responsible for aPKC activation.

**Effects of Mutagenesis of Pseudosubstrate Arginine Residues versus DAG Pocket Arginine Residues on the Activity of His<sub>6</sub>-tagged Forms of PKC- $\iota$ : Plasmid Studies**—We questioned whether the cluster of five basic arginine residues common to pseudosubstrate sequences of all aPKCs  $\zeta/\lambda$ , *viz.* the nearly identical sequences of SIYRRGARRWRKLYCANG for PKC- $\iota/\lambda$  and SIYRRGARRWRKLYRANG for PKC- $\zeta$ , may be important for maintaining aPKCs in an inactive state and for activation by PIP<sub>3</sub>. For this purpose, we thought it important to compare these five arginine residues of the PKC- $\iota/\lambda$  pseudosubstrate sequence to another cluster of nearby arginine residues in the regulatory domain of aPKC that are known to have “activating potential.” Accordingly, we chose four arginine residues that reportedly (13) form a positively charged ring that denies DAG access to a pocket containing a putative DAG-binding site located in the C1 region of the regulatory domain 14–27 residues beyond the pseudosubstrate sequence. Note that the four arginine residues that comprise this protective ring are present in the C1 regions of both PKC- $\iota/\lambda$  and PKC- $\zeta$ , therefore preventing activation of aPKC by DAG. In contrast, they are absent in conventional and novel PKCs, therefore, presumably, allowing an activation by DAG (13). Also note that the removal of these four arginine residues from aPKC has been reported to allow DAG activation of this mutagenized aPKC (13). Therefore, we mutated arginine residues Arg-126, Arg-127, Arg-130, Arg-131, and Arg-133 in the pseudosubstrate

region and arginine residues Arg-147, Arg-150, Arg-151, and Arg-160 in the DAG pocket ring area of His<sub>6</sub>-tagged PKC- $\iota$  to neutral alanine residues. After transfection and expression of plasmids containing these mutant constructs and wild-type His<sub>6</sub>-tagged PKC- $\iota$  in 3T3/L1 adipocytes, the wild-type and mutated forms of His<sub>6</sub>-tagged PKC- $\iota$  were harvested on Ni-NTA-coated beads, eluted, and assayed for aPKC activity. As seen in the representative immunoblot shown in Fig. 3 and as confirmed by the finding of similar maximal kinase activities, it is clear that comparable amounts of enzyme were recovered in the harvested aPKCs and assayed subsequently.

Following mutation of the five arginine residues of the aPKC pseudosubstrate sequence, the resultant mutant, His<sub>6</sub>-R126A/R127A/R130A/R131A/R133A-PKC- $\iota$ , was constitutively active, as judged by apparently maximal increases in “basal” activity and the failure of either insulin treatment in intact 3T3/L1 adipocytes or PIP<sub>3</sub> *in vitro* (*i.e.* added directly to the enzyme assays) to further activate this mutant PKC- $\iota$  (Fig. 3). Moreover, mutagenesis of even a single arginine residue at either Arg-126 or Arg-131 (*i.e.* sites that could flank a central amino acid sequence that can harbor threonine or serine residues that can be phosphorylated) similarly yielded constitutively active PKC- $\iota$  mutants (Fig. 3). In marked contrast, mutagenesis of all four arginine residues in the ring surrounding the pocket containing a putative DAG activation site, *i.e.* His<sub>6</sub>-R147A/R150A/R151A/R160A-PKC- $\iota$ , had no effect on either the basal aPKC activity of this mutant or on the ability of either insulin in intact cells or PIP<sub>3</sub> *in vitro* to activate this mutant (Fig. 3).

**Effects of Mutagenesis of Arginine Residues in the Pseudosubstrate versus DAG Pocket Sequences on Kinase and Functional Activities of His<sub>6</sub>-tagged Forms of PKC- $\iota$ : Adenovirus Studies**—To examine the functional consequences in intact adipocytes of alterations in arginine residues in the pseudosubstrate sequence, compared with alterations of arginine residues in the



**FIGURE 3. Effects of mutation of arginine residues in the pseudosubstrate sequence of PKC- $\iota$  (R126A, R127A, R130A, R131A, and R133A) versus mutation of arginine residues contained within a ring surrounding a putative diacylglycerol activation pocket of PKC- $\iota$  (R147A, R150A, R151A, and R160A) on resting/basal aPKC activity and its activation by insulin *in vivo* and PIP<sub>3</sub> *in vitro*.** Plasmids encoding WT and mutated forms of His<sub>6</sub>-tagged PKC- $\iota$  were transfected into 3T3/L1 adipocytes, and, after allowing 48–72 h for expression, cells were treated for 30 min with 100 nM insulin or left untreated. After incubation, WT and mutated forms of His<sub>6</sub>-tagged PKC- $\iota$  were adsorbed from cell lysates onto Ni-NTA beads from which the His<sub>6</sub>-tagged aPKC was eluted and assayed for aPKC activity with or without maximally effective 10  $\mu$ M PIP<sub>3</sub> as indicated. Immunoblot analysis shows nearly equal expression of the wild-type and mutated forms of His<sub>6</sub>-tagged PKC- $\iota$  as recovered in eluates of Ni-NTA beads. Data are mean  $\pm$  S.E. of duplicate incubations in a representative experiment. Comparable results were obtained in two separate experiments.

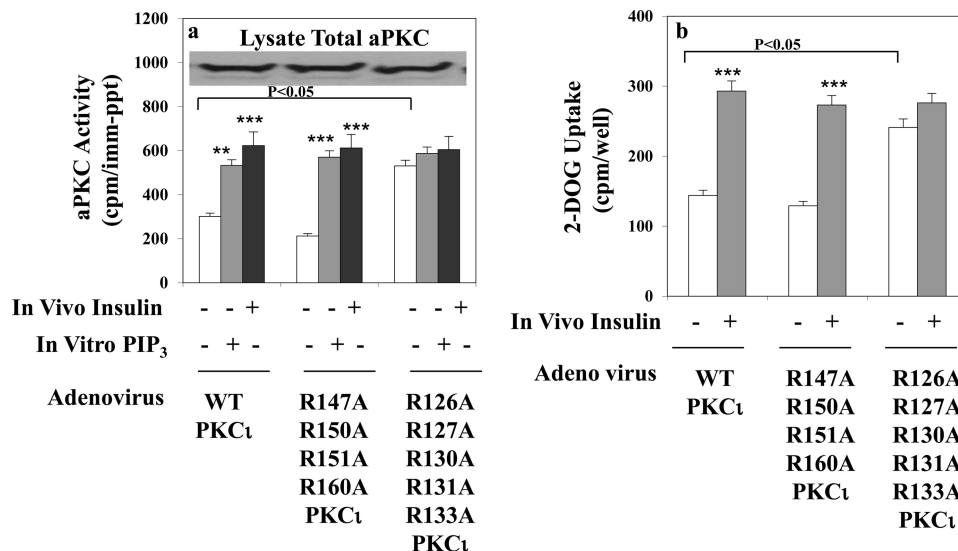
ring area surrounding the putative DAG activation site, the plasmid constructs described above were placed into adenoviruses and inserted into 3T3/L1 adipocytes. In adipocytes infected with either an adenovirus encoding wild-type His<sub>6</sub>-tagged PKC- $\iota$  or an adenovirus encoding His<sub>6</sub>-tagged-PKC- $\iota$  mutated in the DAG pocket ring, *i.e.* the R147A/R150A/R151A/R160A mutant, insulin in intact adipocytes and PIP<sub>3</sub> *in vitro* provoked 2- to 3-fold increases in total immunoprecipitable aPKC activity (Fig. 4*a*). Moreover, insulin provoked comparable 2- to 3-fold increases in glucose transport in intact adipocytes (Fig. 4*b*). In contrast, in adipocytes infected with an adenovirus encoding His<sub>6</sub>-tagged PKC- $\iota$  mutated in the pseudosubstrate sequence, *i.e.* the R126A/R127A/R130A/R131A/R133A mutant, basal aPKC activity (Fig. 4*a*) and glucose transport activity (Fig. 4*b*) were increased constitutively to the same level as that provoked by insulin in adipocytes infected with wild-type His<sub>6</sub>-tagged PKC- $\iota$ , and the addition of insulin in intact adipocytes or PIP<sub>3</sub> *in vitro* was without further effect on either total immunoprecipitable aPKC activity (Fig. 4*a*) or glucose transport (Fig. 4*b*).

**PIP<sub>3</sub> Reverses Exogenous Pseudosubstrate-induced Inhibition of Constitutively Active His<sub>6</sub>-R126A/R127A/R130A/R131A/R133A-PKC- $\iota$** —As shown in Fig. 3, PIP<sub>3</sub> had no effect on the kinase activity of the His<sub>6</sub>-R126A/R127A/R130A/R131A/R133A-PKC- $\iota$  mutant used in Fig. 5, which was constitutively active because of the replacement of all five arginine residues in the endogenous pseudosubstrate sequence. However, with the addition of exogenous pseudosubstrate to incubation of the

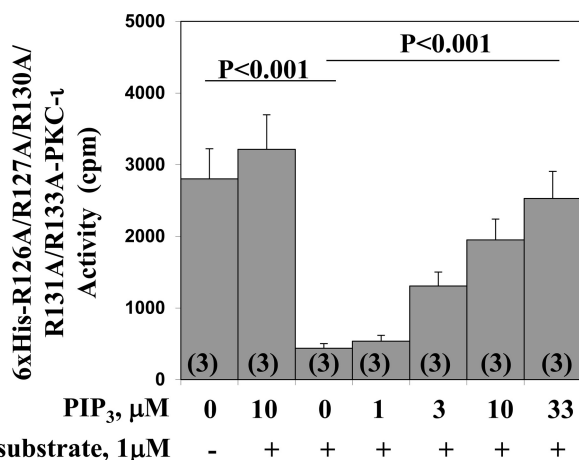
His<sub>6</sub>-R126A/R127A/R130A/R131A/R133A-PKC- $\iota$  mutant, the kinase activity of this mutant was reduced markedly (Fig. 5). Therefore, it was particularly interesting to find that, in this exogenous pseudosubstrate-inhibited state, the addition of PIP<sub>3</sub> led to a dose-dependent activation to levels approaching that of the original constitutive mutant (Fig. 5).

**PIP<sub>3</sub> Reverses Exogenous Pseudosubstrate-induced Inhibition of Full-length Native Forms of Mouse Liver aPKC**—In addition to using PIP<sub>3</sub> to reverse the inhibitory effects of exogenous pseudosubstrate on the His<sub>6</sub>-R126A/R127A/R130A/R131A/R133A-PKC- $\iota$  mutant, we employed a comparable approach with activated forms of native aPKC immunoprecipitated from lysates of mouse liver (which contains comparable amounts of PKC- $\lambda$  and PKC- $\zeta$ ) activated *in vivo* by insulin or *in vitro* by direct addition of 100  $\mu$ M ceramide. As seen in Fig. 6, insulin treatment *in vivo* provoked a strong increase in phosphorylation of the aPKC autophosphorylation site, Thr-563/560-PKC- $\lambda/\zeta$ , that was comparable with that elicited by direct addition of PIP<sub>3</sub> to the assay. Addition of PIP<sub>3</sub> did not elicit a significant increase in auto(trans)phosphorylation above that induced by insulin alone. Similarly, low to high picomolar concentrations of ceramide provoked increases in phosphorylation of Thr-563/560-PKC- $\lambda/\zeta$  comparable with that elicited by insulin and/or PIP<sub>3</sub> (Fig. 6). Further evidence that aPKC was maximally or near maximally activated by these insulin and ceramide treatments was confirmed by the finding that the addition of exogenous PIP<sub>3</sub> *in vitro* (in the absence of added pseudosubstrate) did not provoke further increases in aPKC activity in aPKC stimulated

## Regulation of aPKC Activity



**FIGURE 4. Effects of mutation of arginine residues in the pseudosubstrate sequence of PKC- $\iota$  (R126A, R127A, R130A, R131A, and R133) versus mutation of arginine residues contained within a ring surrounding a putative diacylglycerol activation pocket of PKC- $\iota$  (R147A, R150A, R151A, and R160A) on resting/basal total cellular aPKC enzyme activity and its activation by insulin *in vivo* or PIP<sub>3</sub> *in vitro* (a) and insulin-stimulated glucose transport in intact 3T3/L1 adipocytes (b). a and b, cells were infected with multiplicity of 10 adenovirus-expressing WT or the indicated mutant forms of PKC- $\iota$ , and, after allowing 72 h for expression, the cells were treated for 30 min with 100 nM insulin or left untreated. b, cells were incubated for another 5 min, during which [<sup>3</sup>H]2-deoxyglucose uptake was measured (see "Experimental Procedures"). a, following incubation, total cellular aPKC was immunoprecipitated from cell lysates with anti-PKC- $\lambda/\zeta$  antiserum, and aPKC activity was measured with or without the addition of maximally effective 10  $\mu$ M PIP<sub>3</sub> as indicated. Data are mean  $\pm$  S.E. of four determinations. As shown in the immunoblot analysis, total cellular aPKC levels were comparable in adipocytes expressing WT and mutant forms of PKC- $\iota$ . Note that 3T3/L1 adipocytes only contain mouse PKC- $\iota$ , which is 98% homologous to human PKC- $\iota$ . \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .**



**FIGURE 5. Dose-related stimulatory effects of PIP<sub>3</sub> on pseudosubstrate-inhibited activity of constitutively active PKC- $\iota$  in which five arginine residues in the pseudosubstrate sequence were replaced by alanine residues.** The constitutive mutant form of PKC- $\iota$ , His<sub>5</sub>-126A/R127A/R130A/R131A/R133A-PKC- $\iota$ , was harvested from plasmid-transfected 3T3/L1 adipocytes as described in Fig. 3 and incubated with or without a maximally effective concentration of pseudosubstrate inhibitor (1  $\mu$ M), the indicated concentrations of PIP<sub>3</sub>, and other components of the aPKC assay. Data are mean  $\pm$  S.E. of the number of determinations shown in parentheses.

with either insulin *in vivo* or ceramide *in vitro* (Fig. 7). In addition to increasing phosphorylation at the auto(trans)phosphorylation site, PIP<sub>3</sub> increased phosphorylation of the activation loop site thr-411/410-PKC- $\lambda/\zeta$  (Fig. 6). Of the greatest importance was the finding that, when these activated mouse liver aPKCs were incubated with exogenous pseudosubstrate, their kinase activities were suppressed markedly and that, regardless of whether insulin or ceramide was used to activate this aPKC, the inhibitory effect of pseudosubstrate on the stimulated aPKC

was largely reversed by PIP<sub>3</sub> in a dose-dependent manner (Fig. 7).

**PIP<sub>3</sub> Reverses Pseudosubstrate-induced Inhibition of the Catalytic Domain of the Truncated  $\Delta$ 1-247-T410A/T560A-PKC- $\zeta$  Mutant**—In addition to using the full-length aPKCs forms described above, we also used a form of aPKC that would not only be fully active in the absence of an activating ligand that operates through the regulatory domain but would also be resistant to activation by loop or autophosphorylation. Accordingly, we used a truncated ( $\Delta$ 1-247) form of aPKC that not only lacks the entire regulatory domain, in which extrinsic activators such as PIP<sub>3</sub> are thought to operate, but that, additionally, contains T410E and T560E; *i.e.* glutamate "PO<sub>4</sub>-mimicking/activating" mutations in the catalytic domain. Use of this construct allowed us to be more certain that the inhibition of aPKC activity afforded by the addition of exogenous pseudosubstrate was most likely attributable to its binding to a site in the catalytic domain, presumably the substrate-binding site, and that, therefore, the PIP<sub>3</sub>-dependent reversal of the inhibitory action of exogenous pseudosubstrate was most likely attributable to the displacement of the pseudosubstrate from the substrate-binding site.

As expected, the  $\Delta$ 1-247/T410E/T560E-PKC- $\zeta$  mutant was constitutively active and could not be activated by PIP<sub>3</sub>. On the other hand, this mutant was inhibited dose-dependently by exogenous pseudosubstrate (Fig. 8). Most importantly, PIP<sub>3</sub> reversed the inhibitory effects of exogenous pseudosubstrate in a dose-dependent manner (Fig. 8).

**PIP<sub>3</sub> Binding to Isolated Pseudosubstrate and Lack Thereof to Arginine-Substituted (R126A and R131A) Pseudosubstrates: NMR Studies**—We used NMR to show direct binding of PIP<sub>3</sub> to the isolated pseudosubstrate peptide. As seen in Fig. 9, the native

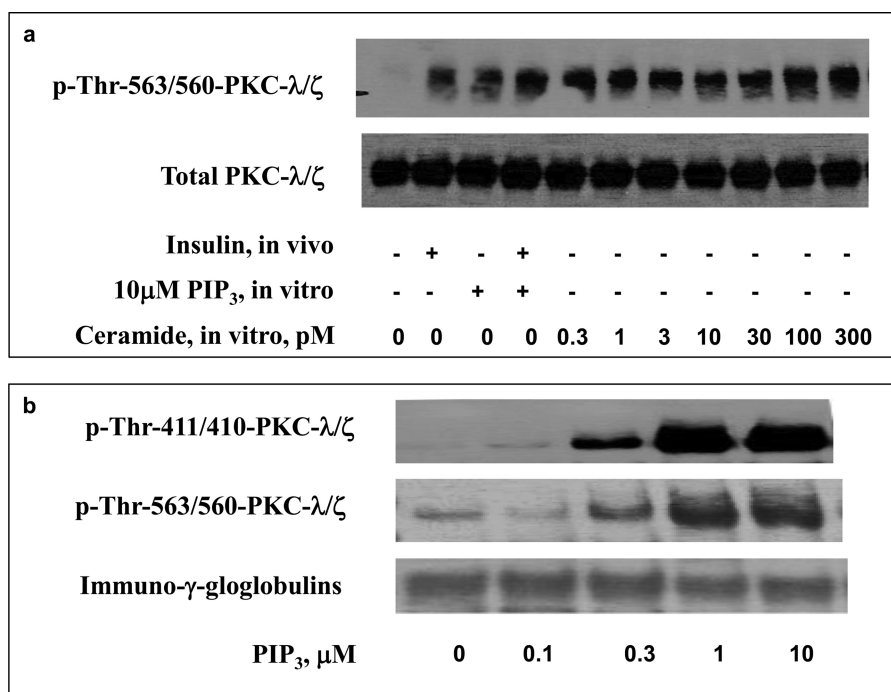


FIGURE 6. **Activation of mouse liver aPKC by treatment with insulin *in vivo* and/or PIP<sub>3</sub> and ceramide *in vitro* (a) and by PIP<sub>3</sub> *in vitro* (b).** *a*, where indicated, mice were treated with vehicle or insulin (1 unit/kg body weight) intraperitoneally 15 min before killing. Liver lysates were obtained, and aPKC was collected by immunoprecipitation and incubated with the indicated concentrations of ceramide and PIP<sub>3</sub> and other components of the aPKC assay. *b*, aPKCs were immunoprecipitated from liver lysates of basal/untreated mice and incubated with the indicated concentrations of PIP<sub>3</sub> and other components of the aPKC assay. After incubation, reaction mixtures were subjected to Western blot analysis for the indicated phosphoproteins and proteins. Representative blots are shown.

pseudosubstrate sequence of PKC- $\iota/\lambda$ , SIYRRGARRWRKLYCANG, yielded an NMR spectrum that was altered (*arrows*) by PIP<sub>3</sub>, indicating binding of PIP<sub>3</sub> to this pseudosubstrate sequence. In contrast, using pseudosubstrate sequences in which either arginine 126 or arginine 131 was replaced by alanine, *i.e.* SIYARGARRWRKLYCANG and SIYRRGARAWRKLYCANG, the ability of PIP<sub>3</sub> to alter the NMR spectrum was lost (Fig. 9). These findings suggested that both arginine 126 and arginine 131 were required for binding of PIP<sub>3</sub> to the pseudosubstrate. This dovetails with the fact that replacement of either arginine 126 or arginine 131 with alanine in the pseudosubstrate led to constitutive activation of aPKC and loss of the ability of PIP<sub>3</sub> to cause further activation, as shown above.

## DISCUSSION

These findings show that arginine residues in the pseudosubstrate sequence play a key role in maintaining aPKC in an inactive state under basal conditions. Indeed, the fact that removal of even a single arginine residue at position Arg-126 or Arg-131 led to full constitutive activation suggests that the maintenance of autoinhibition by a suitably tight interaction between the pseudosubstrate in the regulatory domain and the substrate-binding site in the catalytic domain requires the participation of at least two arginine contact sites that flank a central amino acid sequence that, in a substrate, could harbor a phosphorylation site. In this regard, note that placement of an acidic PO<sub>4</sub>-mimicking aspartate residue between the arginine residues Arg-126 and Arg-131, *i.e.* mimicking a phosphorylated aPKC substrate that should theoretically abrogate binding of the mutated pseudosubstrate to the substrate-binding site, does in fact lead to constitutive activation of PKC- $\zeta$  (20).

The specificity of arginine residues in the pseudosubstrate sequence as critical regulators of aPKC activity is suggested by the finding that the loss of all four arginine residues that form a functional ring that reportedly (13) denies DAG access to a potential activation site had no effect on basal aPKC activity or its activation *in vivo* by insulin, which increases endogenous PIP<sub>3</sub> production, or its activation *in vitro* by direct addition of exogenous PIP<sub>3</sub> to the assay. We did not evaluate other arginine sites to see whether they influence aPKC activity because it appeared that the arginine residues in the pseudosubstrate sequence were sufficient to satisfy the requirements for both autoinhibition and, as discussed below, activation of aPKC by PIP<sub>3</sub>.

In addition to providing evidence that arginine residues in the pseudosubstrate are required for autoinhibition of aPKC, these findings provide strong evidence that PIP<sub>3</sub> activates aPKC by counteracting arginine-dependent autoinhibitory effects of the pseudosubstrate. The most likely explanation for this activating effect is that the acidic D3-PO<sub>4</sub> group of PIP<sub>3</sub> binds to basic arginine residues in the pseudosubstrate sequence and causes their dissociation from acidic residues in the substrate-binding site. The strongest evidence for this suggestion was perhaps our findings in studies of the  $\Delta$ 1-247/T410E/T560E-PKC- $\zeta$  mutant in which PIP<sub>3</sub> had no activating effect unless the activity of this construct was inhibited by addition of pseudosubstrate. In this pseudosubstrate-inhibited state, PIP<sub>3</sub> was a very effective activator of this  $\Delta$ 1-247/T410E/T560E-PKC- $\zeta$  mutant, despite the absence of any regulatory domain components other than the added pseudosubstrate. The most reasonable conclusion

## Regulation of aPKC Activity

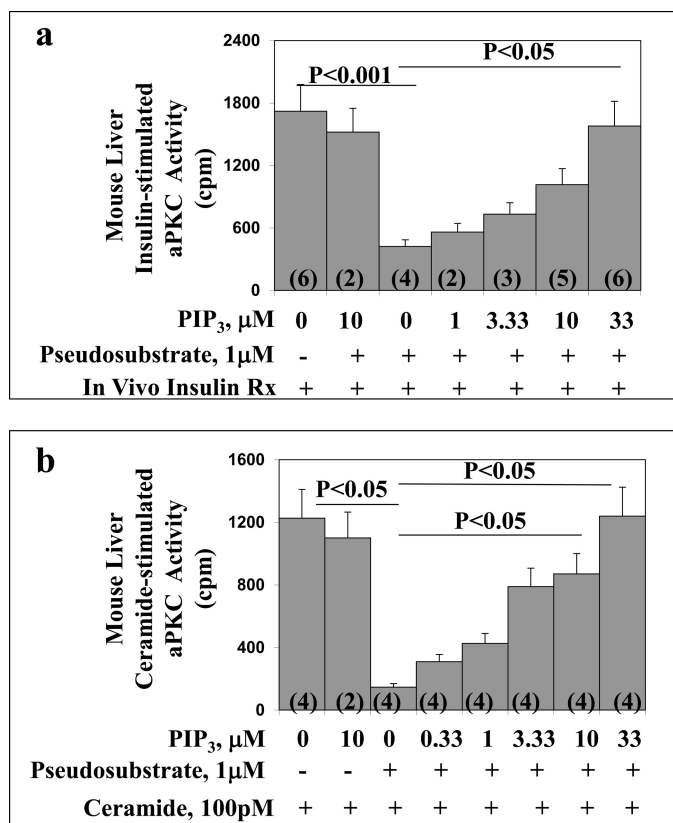


FIGURE 7. Dose-related stimulatory effects of PIP<sub>3</sub> on pseudosubstrate-inhibited activity of mouse liver aPKC activated *in vivo* by insulin (panel a) or *in vitro* by ceramide (panel b). In panel a, liver was obtained from mice treated *in vivo* with insulin (1 units/kg body weight) given intraperitoneally 15 min before killing. Rx, treatment. In panel b, liver was obtained from basal/untreated mice and activated during subsequent *in vitro* assay by addition of maximally effective 100 μM ceramide-16:0. In both cases, total aPKC was immunoprecipitated and assayed in the presence of maximally effective 1 μM pseudosubstrate and indicated concentrations of PIP<sub>3</sub>. Values are mean ± S.E. of the number of determinations shown in parentheses.

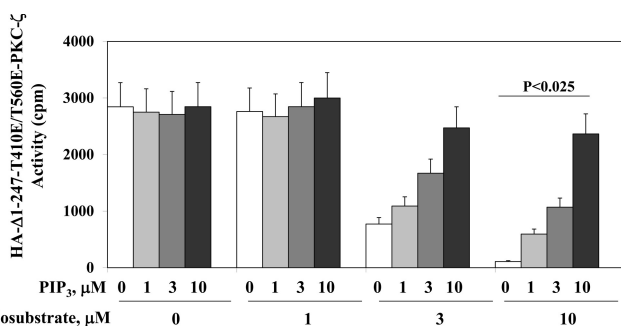


FIGURE 8. Dose-related stimulatory effects of PIP<sub>3</sub> on pseudosubstrate-inhibited activity of a truncated, glutamate-pseudophosphorylated, constitutively active PKC-ζ. 3T3/L1 adipocytes were grown for 72 h with a plasmid encoding HA-tagged Δ1-247-T410E/T560E-PKC-ζ, and then the PKC-ζ mutant was harvested with anti-HA antiserum and incubated with increasing concentrations of pseudosubstrate to diminish activity of the constitutive Δ1-247-T410E/T560E-PKC-ζ mutant and increasing concentrations of PIP<sub>3</sub> to counteract the inhibitory effects of the pseudosubstrate. Data are mean ± S.E. of two to three determinations.

for this finding is that the autoinhibitory pseudosubstrate is targeted by PIP<sub>3</sub>.

Further support for the idea that PIP<sub>3</sub> activates aPKC by reversing pseudosubstrate-dependent autoinhibition was our finding that stimulatory effects of PIP<sub>3</sub> were seen only in con-

junction with pseudosubstrate-induced inhibition of the full-length His<sub>6</sub>-R126A/R127A/R130A/R131A/R133A-PKC-ι mutant, which is otherwise constitutively active as a result of loss of all five arginine residues in its pseudosubstrate sequence. In addition, the fact that similar stimulatory effects of PIP<sub>3</sub> were seen following exogenous pseudosubstrate-induced inhibition of insulin- and ceramide-activated, full-length forms of mouse liver aPKC suggests that the findings seen with the two constitutively active mutants, *viz.* His<sub>6</sub>-R126A/R127A/R130A/R131A/R133A-PKC-ι and Δ1-247/T410E/T560E-PKC-ζ, were not particular to these mutants and could be seen in native aPKC forms. This confirmation seemed important because, for uncertain reasons, higher concentrations of pseudosubstrate were needed to inhibit the truncated Δ1-247/T410E/T560E-PKC-ζ as compared with activated full-length forms of aPKC.

It was interesting to find that the addition of PIP<sub>3</sub> to the *in vitro* assay of aPKC immunoprecipitated from control (unstimulated) mouse liver provoked increases in the phosphorylation of the activation loop site, Thr-411/410-PKC-λ/ζ, and the auto(trans)phosphorylation site, Thr-563/560-PKC-λ/ζ, which are required for, and indicative of, activation (11, 12). This demonstrates that the allosteric alteration of aPKC (presumably molecular unfolding because of pseudosubstrate dissociation from the substrate-binding site) induced by PIP<sub>3</sub> can precede and trigger these phosphorylations. In this regard, it is noteworthy that PDK1 is known to be present in aPKC immunoprecipitates (21).

It is interesting that findings in crystallographic and solution structure studies suggest that basic arginine and lysine residues within the PH domains of both Akt and PDK1 bind to the acidic D3-PO<sub>4</sub> groups of PIP<sub>3</sub> and phosphatidylinositol-3,4-(PO<sub>4</sub>)<sub>2</sub> (22, 23). This interaction between acidic phosphoinositides and basic residues in the PH domains of Akt and PDK1 is similar to what appears to occur during PIP<sub>3</sub>-induced aPKC activation, *viz.* an interaction between the acidic D3-PO<sub>4</sub> group of PIP<sub>3</sub> and one or more of the basic arginine residues in the pseudosubstrate sequence. In this regard, it is noteworthy that phosphatidylinositol-4,5-(PO<sub>4</sub>)<sub>2</sub> is much less effective than PIP<sub>3</sub> for activating Akt and aPKC (6, 11, 12) and that it seems likely that the D3-PO<sub>4</sub> group of PIP<sub>3</sub> largely accounts for its activating properties.

Relative to these findings, Lopez-Garcia *et al.* (24) have suggested that, along with the attachment of the pseudosubstrate to the substrate-binding site, there is an additional mechanism for inhibition of aPKC activity that involves binding of the C1 region to the C-terminal PIF-pocket. In addition, Graybill *et al.* (25) have suggested that a region of C1 just beyond the pseudosubstrate sequence is required along with the pseudosubstrate for autoinhibition of aPKC and, interestingly, that this C1 region is targeted by Par-6 and, thereby, activates aPKC by a mechanism involving displacing the endogenous pseudosubstrate. Accordingly, it appears that there is more than one mechanism for activation of aPKC. By analogy, it may be hypothesized that the activation of aPKC by ceramide, details of which are currently uncertain but appear to involve direct binding to aPKC (26), is mediated by an interaction with a region of C1 that induces displacement of the pseudosubstrate. In any case, here we show that ceramide-induced activation can be overcome by addition of an exogenous pseudosubstrate-con-



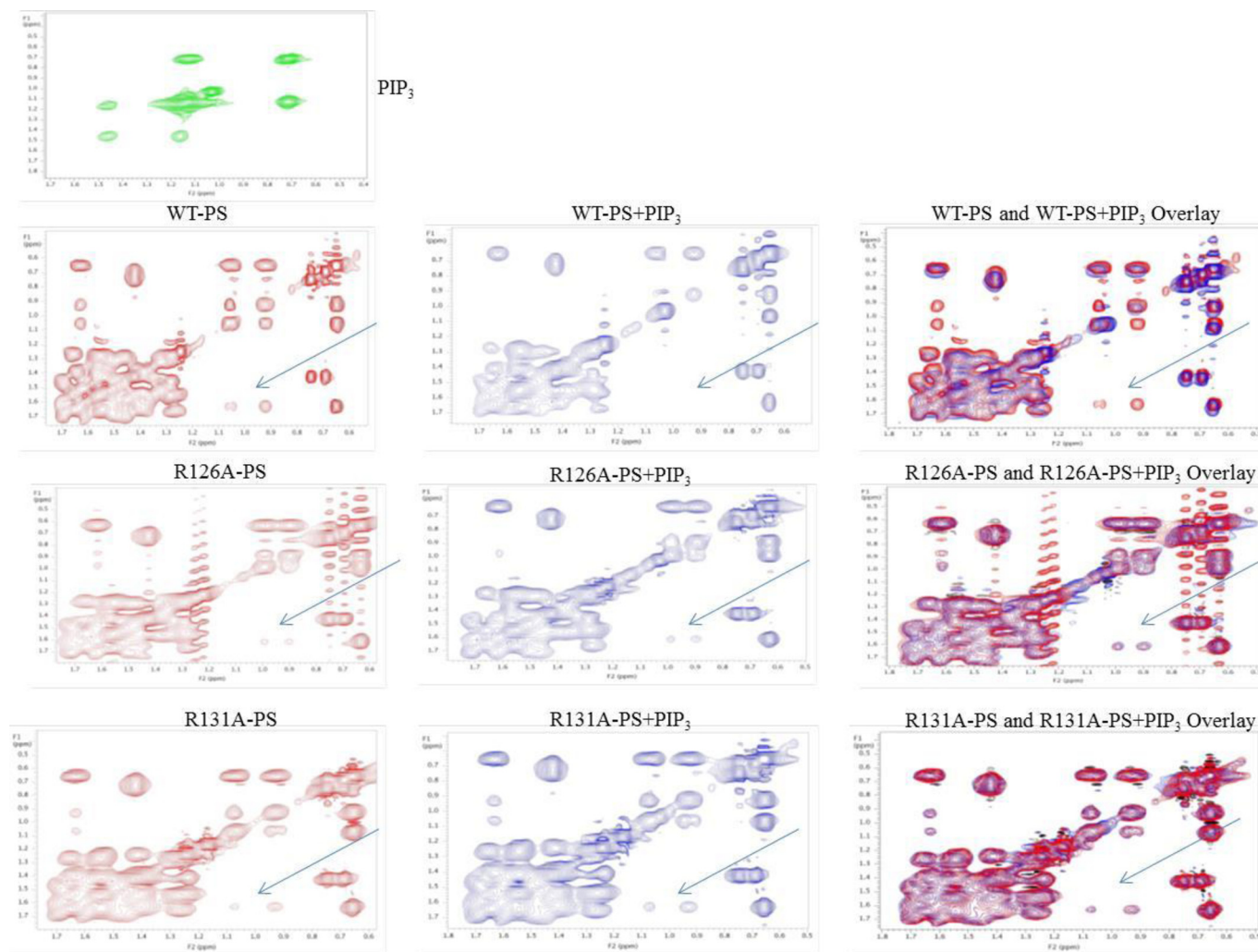


FIGURE 9. Shown are the effects of PIP<sub>3</sub> on the NMR spectra of WT pseudosubstrate (PS) peptide (SIYRRGARRWRKLYCANG) and arginine-replaced pseudosubstrate peptide sequences (SIYARGARRWRKLYCANG and SIYRRGARAWRKLYCANG); i.e. alanine substituted for arginine at Arg-126 and Arg-131 (R126A and R131A). Peptides were analyzed without (left column) or without PIP<sub>3</sub> (center column). The right column shows merged spectra. Also shown is the NMR spectrum of PIP<sub>3</sub>.

taining peptide and that this inhibitory effect of exogenous pseudosubstrate can be reversed by PIP<sub>3</sub>.

To summarize, these findings show that removal of arginine residues from the pseudosubstrate sequence of PKC- $\iota$  by site-directed mutagenesis leads to constitutive activation and loss of ability of PIP<sub>3</sub> to further activate the mutagenized aPKC. These findings also show that, with addition of exogenous pseudosubstrate to inhibit the constitutively active form of PKC- $\iota$  that lacks all five arginine residues in the pseudosubstrate sequence, *viz.* His<sub>6</sub>-R126A/R127A/R130A/R131A/R133A-PKC- $\iota$ , fully activated forms of full-length native mouse liver aPKC, and a constitutively active, truncated mutant form of PKC- $\zeta$  that lacks all regulatory domain elements and, additionally, cannot be phosphorylated at loop or autophosphorylation sites, *viz.*  $\Delta$ 1–247/T410E/T560E-PKC- $\zeta$ , the addition of PIP<sub>3</sub> resulted in nearly full activation. These findings suggest that basic arginine residues in the pseudosubstrate sequence of the regulatory domain, by presumably binding to acidic residues in the substrate-binding site in the catalytic domain, maintain aPKC in a folded, inactive state and, moreover, are targeted by PIP<sub>3</sub> for

displacement from the substrate-binding site during enzyme activation.

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