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Group VIA Phospholipase A_2 Forms a Signaling Complex with the Calcium/Calmodulin-dependent Protein Kinase IIβ Expressed in Pancreatic Islet β-Cells*

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Abstract

Insulin-secreting pancreatic islet β-cells express a Group VIA Ca^{2+}-independent phospholipase A_2 ($iPLA_2\beta$) that contains a calmodulin binding site and protein interaction domains. We identified Ca^{2+}/calmodulin-dependent protein kinase IIβ (CaMKIIβ) as a potential $iPLA_2\beta$-interacting protein by yeast two-hybrid screening of a cDNA library using $iPLA_2\beta$ cDNA as bait. Cloning CaMKIIβ cDNA from a rat islet library revealed that one dominant CaMKIIβ isoform mRNA is expressed by adult islets and is not observed in brain or neonatal islets and that there is high conservation of the isoform expressed by rat and human β-cells. Binary two-hybrid assays using DNA encoding this isoform as bait and $iPLA_2\beta$ DNA as prey confirmed interaction of the enzymes, as did assays with CaMKIIβ as prey and $iPLA_2\beta$ bait. His-tagged CaMKIIβ immobilized on metal affinity matrices bound $iPLA_2\beta$, and this did not require exogenous calmodulin and was not prevented by a calmodulin antagonist or the Ca^{2+} chelator EGTA. Activities of both enzymes increased upon their association, and $iPLA_2\beta$ reaction products reduced CaMKIIβ activity. Both the $iPLA_2\beta$ inhibitor bromoenol lactone and the CaMKIIβ inhibitor KN93 reduced arachidonate release from INS-1 insulinoma cells, and both inhibit insulin secretion. CaMKIIβ and $iPLA_2\beta$ can be coimmunoprecipitated from INS-1 cells, and forskolin, which amplifies glucose-induced insulin secretion, increases the abundance of the

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immunoprecipitatable complex. These findings suggest that iPLA$_2$β and CaMKIIβ form a signaling complex in β-cells, consistent with reports that both enzymes participate in insulin secretion and that their expression is coinduced upon differentiation of pancreatic progenitor to endocrine progenitor cells.

Phospholipases A$_2$ (PLA$_2$)[1] are a diverse group of enzymes that catalyze hydrolysis of *sn*-2 fatty acid substituents from glycerophospholipid substrates to yield a free fatty acid and a 2-lysophospholipid (1). The Group VIA PLA$_2$ designated iPLA$_2$β has a molecular mass of 84–88 kDa and does not require Ca^{2+} for catalysis (2). Various splice variants of iPLA$_2$β are expressed at high levels in testis (3), brain (4), and pancreatic islet β-cells (5), among other tissues.

Certain nutrients, hormones, neurotransmitters, and pharmacologic agents stimulate insulin secretion from β-cells, and the dominant physiologic insulin secretagogue is D-glucose. A series of signals that result from glucose-induced ATP production and alterations of intracellular redox potentials trigger insulin secretion via a rise in cytosolic [Ca^{2+}], and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is an important β-cell [Ca^{2+}] sensor and mediator of Ca^{2+}-dependent events in insulin secretion (6–11). Much evidence (12–22) suggests that iPLA$_2$β also participates in insulin secretion, including the facts that the mechanism-based bromoenol lactone (BEL) inhibitor of iPLA$_2$β suppresses glucose-induced hydrolysis of arachidonate from islet membrane phospholipids, the rise in β-cell cytosolic [Ca^{2+}], and insulin secretion (19–22).

Depleting intracellular Ca^{2+} stores activates iPLA$_2$β in β-cells and vascular smooth muscle cells (23, 24), and iPLA$_2$β participates in store-operated entry of Ca^{2+} from the extracellular space (25), which is thought to be involved in glucose-induced insulin secretion (26–31). Regulating store-operated calcium (SOC) entry requires that intracellular Ca^{2+} stores communicate with plasma membrane ion channels, and calmodulin participates in cross-talk between Ca^{2+} stores and SOC channels (25, 32). Lipid signaling molecules (*e.g.* lysophospholipids) and Ca^{2+}-sensitive kinases and phosphatases (*e.g.* CaMKIIβ and calcineurin) are also proposed to affect these interactions (9, 10, 25, 32). Mechanisms whereby iPLA$_2$β participates in glucose-induced rises in β-cell cytosolic [Ca^{2+}] and insulin secretion are likely to involve Ca^{2+}-sensitive regulation of modulatory and effector proteins by phosphorylation-dephosphorylation events (9, 10), and iPLA$_2$β activity is also affected by local [Ca^{2+}] increments that relieve its tonic inhibition by Ca^{2+}/calmodulin (2, 8, 25).

The amino acid sequence of iPLA$_2$β contains an ankyrin repeat domain with eight strings of a repetitive motif of about 33 amino acid residues each (34). Ankyrin repeats link integral membrane proteins to the cytoskeleton and mediate protein-protein interactions in signaling (34–38). Ankyrin binds to inositol trisphosphate receptors (37), for example, which are located on Ca^{2+}-containing vesicles that release intracellular Ca^{2+} when β-cells are stimulated with glucose (26–31). Ankyrin G also associates with skeletal muscle postsynaptic membranes and sarcoplasmic reticulum (38), and CaMKII participates in regulating local [Ca^{2+}] gradients in subcellular zones involved in Ca^{2+} signaling. CaMKII is an important Ca^{2+} signaling effector and serves as a gauge that temporally integrates [Ca^{2+}] signal intensities (39), and calmodulin participates in several Ca^{2+}-dependent processes in insulin secretion by β-cells (40, 41). Calmodulin and iPLA$_2$β interact functionally (2, 8, 23, 24, 33), and the iPLA$_2$β domain from residues 650–722 contains a calmodulin binding site (2).

[1]The abbreviations used are: PLA$_2$, phospholipase A$_2$; BEL, (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one; CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; iPLA$_2$, calcium-independent phospholipase A$_2$; Pipes, 1,4-piperazinediethanesulfonic acid; SOC, store-operated calcium channel; X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

During cell signaling, $iPLA_2$ translocates to membranes (22, 25, 32) where it interacts with regulatory proteins to effect cellular activation. To identify proteins that interact with $iPLA_2\beta$ to understand better its role in signaling, we performed yeast two-hybrid screening and have found that $iPLA_2\beta$ interacts with the specific CaMKIIβ isoform expressed in pancreatic islet β-cells. This interaction is demonstrated by multiple independent techniques, and the interaction affects both $iPLA_2\beta$ and CaMKIIβ activities, thereby defining a signaling complex.

EXPERIMENTAL PROCEDURES

Materials

The materials [γ-^{32}P]ATP, 55 mCi/mmol (16:0/[^{14}C]18:2)-glycerophosphocholine [1-palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine, rainbow molecular mass standards, enhanced chemiluminescence (ECL) reagent, and [^{3}H]arachidonic acid were obtained from Amersham Biosciences. SDS-PAGE supplies were purchased from Bio-Rad. Coomassie reagent was obtained from Pierce. Alkaline phosphatase and peroxidase-conjugated goat anti-rabbit IgG antibodies were obtained from Roche Applied Science. Protease inhibitor mixture, kanamycin, ampicillin, ATP, calmodulin, autocamtide-3, arachidonic acid, lysophosphatidylcholine, calmodulin kinase II inhibitor KN93, common reagents, and salts were obtained from Sigma. Tetracycline was obtained from Invitrogen. Gentamicin and cell culture media were obtained from the Tissue Culture Support Center (Washington University, St. Louis, MO). TALON metal affinity resin, a rat brain cDNA library, AH109 yeast cells, and media for yeast two-hybrid screening were obtained from Clontech (Palo Alto, CA). Polyclonal antibodies to $iPLA_2\beta$ and CaMKII were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The calmodulin inhibitor W13 was obtained from Calbiochem. The $iPLA_2\beta$ suicide substrate BEL was obtained from Cayman Chemical Company (Ann Arbor, MI). Avian myeloblastosis virus reverse transcriptase was obtained from Roche Applied Science.

Screening of a Rat Brain cDNA Library in the Yeast Two-hybrid System

A rat brain cDNA library cloned into pACT2 vector (containing the *LEU2* gene for selection) to produce fusion between protein-encoding DNA sequences and the DNA activation domain of GAL4 was used as prey. To produce the bait construct, full-length $iPLA_2\beta$ cDNA cloned from rat pancreatic islets was ligated into the SalI-EcoRI sites of the two-hybrid BD pAS2-1 vector, which contained a *TRP1* gene for selection, resulting in in-frame fusion of $iPLA_2\beta$ with the DNA binding domain of the yeast GAL4 protein. The fidelity of constructs was confirmed by automated sequencing. The yeast strain AH109 was used for screening assays, and this strain contains *HIS3* and *lacZ* reporter genes. Expression of each of these genes is regulated by a distinct GAL4-responsive promoter under control of a GAL4-responsive upstream activation site. Lack of autonomous activation by the $iPLA_2\beta$/DNA binding domain fusion product was demonstrated by plating cells transformed with bait alone on media lacking histidine. In these assays, both bait and prey plasmids were transformed simultaneously into AH109 yeast cells, which were plated on medium lacking leucine, tryptophan, and histidine and allowed to grow at 30 °C for 4 days. Putative positive colonies were lifted onto filter paper and incubated with the chromogenic substrate X-gal. Interactions were confirmed when the blue β-galactosidase reaction product was evident after 4 h of incubation at room temperature. Plasmids were then recovered from yeast and transformed into DH5α bacterial cells using ampicillin for selection. Isolated plasmids were sequenced, and BLAST searches were performed against GenBank (NIH genetic sequence data base) to identify putative $iPLA_2\beta$-interacting proteins.

Molecular Cloning of CaMKIIβ cDNA from a Rat Islet Library

Total RNA was isolated from adult rat islets as described previously (5). First strand cDNA was transcribed with avian myeloblastosis virus reverse transcriptase. PCR was performed using a pair of gene-specific primers designed from regions of cDNA sequence that are conserved in the mouse and rat brain CaMKIIβ cDNA sequences (sense, 5′-ATCGCCACCGCCATGGCCACC-3′; antisense, 5′-CAGGCGCAGCTCTCACTGCAG-3′). A PCR band of 1,650 bp was gel purified, ligated into pGEM-T vector, and transformed into DH5α cells for amplification. DNA was purified and sequenced using T3 and T7 primers and gene-specific primers.

Binary Yeast Two-hybrid Assays

The iPLA$_2$β cDNA was cloned from an adult rat islet library (5). Full-length iPLA$_2$β cDNA was ligated into BD vector pAS2-1 or AD vector pACT2 and used as bait or prey. Full-length CaMKIIβ cDNA was cloned into the AD vector pACT2 or BD vector pAS2-1 and used as prey or bait. Both bait and prey plasmids were transformed simultaneously into AH109 yeast cells, which were plated on restriction medium. After incubation (30 °C, 4 days), colonies were lifted onto filter paper and screened as described above. Colonies that produced the blue β-galactosidase reaction product were considered positive for the interaction between iPLA$_2$β and CaMKIIβ.

Cloning and Expression of His-tagged CaMKIIβ, His-tagged iPLA$_2$β, and FLAG-tagged Proteins in Sf9 Cells

Recombinant proteins were expressed in *Spodoptera frugiperda* (Sf9) cells using the Bac-to-Bac baculovirus expression system (Invitrogen) following the manufacturer's instructions, as described in detail elsewhere (2, 23, 33, 42). cDNA containing the entire coding sequence of His-tagged CaMKIIβ, His-tagged iPLA$_2$β, or FLAG-tagged iPLA$_2$β was cloned into the SalI-EcoRI site of the pFastBac-1 vector. The sequence of the insert was verified, and the plasmid was then transformed into DH10Bac cells. Recombinant bacmid DNA was isolated using an alkaline lysis protocol modified for high molecular weight plasmid purification. PCR analysis was performed with purified bacmid DNA and pUC/M13 forward and reverse primers to characterize the inserts in the recombinant bacmid DNA. The recombinant baculovirus was produced by transfecting the recombinant bacmid DNA into Sf9 cells. The baculovirus was amplified and used to infect Sf9 cell cultures to express the recombinant proteins (2, 23, 33, 42).

Immunoblotting Analyses

Proteins were analyzed by SDS-PAGE and transferred to a nylon membrane that was subsequently blocked with 5% nonfat dry milk for 1 h. The membrane was washed and incubated for 1 h with polyclonal antibody (1:200) to iPLA$_2$β or CaMKII. The membrane was then incubated with secondary antibody (1:30,000) coupled to horseradish peroxidase, and the antibody complex was visualized by ECL.

Interaction of CaMKIIβ with iPLA$_2$β and Protein Pull-down Assays

In some experiments, both iPLA$_2$β and His-tagged CaMKIIβ proteins were coexpressed in Sf9 cells. The Sf9 cell cytosol containing iPLA$_2$β and His-tagged CaMKIIβ proteins was mixed with TALON metal affinity resin in the presence or absence of added Ca^{2+}/calmodulin, the calmodulin antagonist W13, or the Ca^{2+} chelator EGTA and incubated (room temperature, with shaking, for 1 h). The mixture was washed with 10 bed volumes of wash buffer (50 mM Na_2HPO_4, 500 mM NaCl, pH 7.8) twice and transferred onto a gravity-flow column. The His-tagged CaMKIIβ was eluted with elution buffer (50 mM Na_2HPO_4, 300 mM NaCl, and 200 mM imidazole, pH 7.8) and collected in 0.5-ml fractions. Desorbed

proteins were visualized by immunoblotting analyses with antibodies to iPLA$_2$β or CaMKIIβ.

In other experiments, iPLA$_2$β protein was first expressed in Sf9 cells and purified as described previously (23, 33). Cytosol was prepared from Sf9 cells infected with baculovirus containing cDNA that encoded His-tagged CaMKIIβ and mixed with 1 ml of TALON metal affinity resin, as described above. The resin was washed and mixed with purified iPLA$_2$β protein. The mixture was then incubated (30 min at room temperature with shaking), washed three times, and loaded onto a 5-ml gravity-flow column. Bound proteins were desorbed with elution buffer, collected in 0.5-ml fractions, and analyzed by immunoblotting with antibodies specific for iPLA$_2$β or CaMKIIβ.

Immunoprecipitation of FLAG-tagged iPLA$_2$β and CaMKIIβ Expressed in Sf9 Cells

Sf9 cells expressing FLAG-tagged iPLA$_2$β, CaMKIIβ, or both were harvested by centrifugation, washed with phosphate-buffered saline, resuspended in cell lysis buffer supplemented with protease inhibitors, and homogenized by sonication. Cytosol was prepared by centrifugation (15,000 × *g*, 20 min) and incubated with 100 μl of anti-FLAG M2 affinity resin (2 h, 4 °C, gentle rotation) in the presence or absence of 10 mM Ca^{2+} chelator EGTA. Immunoprecipitated material was recovered by centrifugation and washed four times with wash buffer. Samples immunoprecipitated with anti-FLAG affinity resin were eluted with elution buffer (0.1 M glycine, pH 3.5). Aliquots (30 μl) were analyzed by 10% SDS-PAGE, transferred onto a nylon membrane, and blotted with iPLA$_2$β or CaMKIIβ antibodies (1:200) followed by horseradish peroxidase-conjugated secondary antibodies (1:30,000).

Enzyme Activity Assays

For CaMKIIβ activity assays, sample buffer (50 mM Pipes, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ATP, 0.75 mM $CaCl_2$, 20 μg/ml calmodulin, 20 μM autocamtide-3, 2 μCi of [γ-^{32}P]ATP, pH 7.4) was mixed with CaMKIIβ (final volume 50 μl) and incubated (30 °C, 3 min). Assays were initiated by adding His-tagged CaMKIIβ and terminated by adding 100 mM EDTA. Aliquots (30 μl) of the mixture were placed on Whatman P-81 phosphocellulose paper, which was washed with 75 mM H_3PO_4 and air-dried. Phosphorylated autocamtide-3 (a CaMKIIβ model substrate) was quantified by liquid scintillation counting of ^{32}P. Control assays were performed without added Ca^{2+}/calmodulin and with 10 mM EGTA.

The iPLA$_2$β activity assays were performed as described previously (5, 22). Briefly, 100 μl of sample was added to assay buffer containing 10 mM EGTA. Reactions were initiated by injecting 5 μl of 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycerol-3-phosphorylcholine (specific activity 50 mCi/mmol, final concentration 5 μM) in ethanol. The assay mixture was incubated (37 °C, 5 min, with shaking), and the reaction was terminated by adding 100 μl of butanol. A 25-μl aliquot of the butanol layer was analyzed by Silica Gel G TLC as described previously (19). The amount of ^{14}C-labeled free fatty acid was determined by liquid scintillation spectrometry.

[^{3}H]Arachidonic Acid Release Measurements

INS-1 insulinoma cells (5 × 10^5 cells/well) were prelabeled for 20 h with 0.5 μCi/ml [^{3}H]arachidonic acid and placed in serum-free medium for 1 h. The cells were washed three times with glucose-free RPMI 1640 medium to remove unincorporated radiolabel. Cells were treated with 20 μM BEL or 8 μM KN93 for 30 min before adding RPMI 1640 medium containing 0.5% bovine serum albumin and incubating for 1 h. The medium was then removed and replaced with fresh medium of identical composition, and the cells were

incubated for 40 min. Supernatants and cells were separated by centrifugation (500 × *g*, 5 min) and assayed for ^{3}H content by liquid scintillation spectrometry.

Coimmunoprecipitation of iPLA$_2$β and CaMKIIβ from INS-1 Cells

Immunoprecipitation was performed with a protein A-agarose slurry that had been washed twice with phosphate-buffered saline, mixed with a 10-μl solution of antibody to CaMKIIβ or to iPLA$_2$β, and incubated (room temperature, 40 min). The mixture was centrifuged, and the supernatant was discarded. The agarose-antibody complex in the precipitate was washed three times with phosphate-buffered saline, mixed with INS-1 cell cytosol, and incubated (overnight, 4 °C, with shaking). Immunoprecipitates were collected by centrifugation, washed, boiled for 5 min in SDS-PAGE sample loading buffer, and analyzed by SDS-PAGE. Proteins were transferred to nylon membranes, and immunoblotting was performed with primary antibody to iPLA$_2$β or to CaMKIIβ and secondary antibody coupled to horseradish peroxidase, as described above.

RESULTS

Yeast Two-hybrid Screening Indicates That CaMKIIβ Is an iPLA$_2$β-interacting Protein

To identify proteins that interact with iPLA$_2$β, a yeast two-hybrid screen of a rat brain cDNA library was performed using iPLA$_2$β cDNA cloned from a rat islet cDNA library as bait. The commercially available rat brain cDNA library was used for screening because there are many biochemical similarities between brain and islets, including high iPLA$_2$β expression (19–22, 34). Colonies were identified that activated transcription of both the *HIS3* gene (permitting autotrophic selection) and the *lacZ* reporter gene (permitting X-gal analysis) in the presence of bait. Such colonies were purified by culture after serial dilution, and the sequences of their cDNA inserts were determined. One colony contained cDNA that encoded 241 residues of rat brain CaMKIIβ N-terminal amino acid sequence (residues 34–274). Several other colonies also contained inserts with the CaMKIIβ sequence. This interaction was examined further because of its likely functional importance, which is suggested by the facts that calmodulin is an important β-cell Ca^{2+}-binding protein (43) and that β-cells express high levels of CaMKIIβ (44, 48), which regulates voltage-operated Ca^{2+} channels involved in insulin secretion (7, 45–47). Insulinoma cell secretion is also potentiated by overexpressing CaMKIIβ (49) or iPLA$_2$β (22), and iPLA$_2$β binds calmodulin (2, 34).

Cloning CaMKIIβ from a Rat Islet cDNA Library Reveals Tissue Specificity and a Developmental Profile of CaMKIIβ Isoform Expression

Pancreatic islets express distinct CaMKII isoforms, and adult rat islets express predominantly the CaMKIIβ isoform (48, 49). To determine whether CaMKII isoform(s) expressed in rat islets, like those in rat brain, also interact with iPLA$_2$β, we cloned CaMKIIβ cDNA from adult rat islets. Reverse transcription-PCR was performed using RNA isolated from rat islets as template and a pair of primers designed from regions of cDNA sequence which are conserved in rat and mouse CaMKIIβ. The PCR product was cloned. Sequencing the insert revealed a putative initiation codon (ATG) at the 5′-end, a stop codon (TGA) at the 3′-end, and the entire coding sequence in an intervening single open reading frame. Fig. 1 illustrates the nucleotide and deduced amino acid sequences of the CaMKIIβ isoform cloned from adult rat islets (ACaMKII). Fig. 2*A* illustrates sequence alignments for CaMKIIβ from rat brain (BCaMKII), adult rat islets (ACaMKII), human β-cells (HCaMKII), and neonatal rat islets (NCaMKII).

The CaMKIIβ cDNA cloned from adult rat islet mRNA contained a complete coding sequence of 1,509 bp which encodes 503 amino acid residues (Fig. 1), and this CaMKIIβ

isoform is distinct from previously (50, 51) described rat isoforms. Analysis of nucleotide sequences revealed that ACaMKIIβ differs from BCaMKIIβ (50) by the lack of sequence corresponding to the first (residues 316–339) and third (residues 379–393) variable domains (Fig. 2*A*). ACaMKIIβ differs from NCaMKIIβ (51) by the absence of the sequence from residues 370 to 456 in the association domain (Fig. 2*B*). Sequence alignments revealed 99.4% amino acid sequence identity between ACaMKIIβ and the HCaMKIIβ isoform cloned from human insulinoma cells (48). The ACaMKIIβ and HCaMKIIβ sequences differ only in 3 amino acid residues in variable domain 2 (Fig. 2*B*).

To search for other subtypes of CaMKIIβ in adult rat islets, we performed a series of reverse transcription-PCR experiments using RNA from adult rat islets as template and primers designed from various regions of the ACaMKIIβ sequence, but we observed no other CaMKIIβ subtype in adult rat islets (not shown). Adult rat pancreatic islets and adult human β-cells thus express mRNA that encodes a CaMKIIβ isoform that differs from those in adult brain or in neonatal islets, and the latter two isoforms also differ from each other. There is thus both tissue specificity and a developmental profile of CaMKIIβ isoform expression, but there is little rat-to-human species heterogeneity in the CaMKIIβ isoform expressed in adult pancreatic islet β-cells.

Binary Yeast Two-hybrid Assays Confirm the Interaction between iPLA$_2$β and CaMKIIβ

To confirm the interaction between iPLA$_2$β and ACaMKIIβ observed in yeast two-hybrid screening experiments, binary yeast two-hybrid assays were performed. We first used ACaMKIIβ as bait and iPLA$_2$β as prey. When bait or prey alone was transformed into yeast cells, no colonies grew in medium lacking leucine, tryptophan, and histidine, but when both bait and prey were transformed simultaneously into yeast cells, colonies formed and produced blue reaction products when treated with the chromogenic substrate X-gal (Fig. 3*B*, *left column*) that reflect interaction between ACaMKIIβ and iPLA$_2$β. When the bait and prey DNA were switched (so that iPLA$_2$β was bait, and ACaMKIIβ was prey), similar results were obtained (Fig. 3*B*, *right column*). These results reflect a specific interaction between iPLA$_2$β and CaMKIIβ.

To identify domains of the proteins essential for their interaction, we performed binary yeast two-hybrid assays using N-or C-terminal fragments of iPLA$_2$β as the bait or prey and N- or C-terminal fragments of CaMKIIβ as the prey or bait. Fig. 3*B* shows the schematic representation of wild-type iPLA$_2$β and CaMKIIβ proteins and of their N- and C-terminal fragments. When the N-terminal fragment of iPLA$_2$β (NiPLA$_2$β) was used as the bait or prey and the N-terminal fragment of CaMKIIβ (NCaMKIIβ) was used as the prey or bait, large colonies formed after incubation at 30 °C for 4 days, and these colonies turned blue after incubation with the chromogenic substrate X-gal for 4 h at room temperature (Fig. 3*C*, *lane 1*). When an N-terminal fragment (NiPLA$_2$β or NCaMKIIβ) was used as bait or prey and a C-terminal fragment (CiPLA$_2$β or CCaMKIIβ)asthe prey or bait, only small colonies formed after incubation at 30 °C for 4 days (Fig. 3*C*, *lanes 2* and *3*). These colonies were lifted onto filter paper and incubated until they grew large enough to perform the X-gal assay. As illustrated in Fig. 3*C* (*lanes 2* and *3*), these colonies failed to turn blue after incubation with the chromogenic substrate X-gal, indicating that the interactions between the C-terminal domains of iPLA$_2$β and CaMKIIβ are weak and nonspecific. No colonies formed when the C-terminal fragment CiPLA$_2$β was used as bait or prey and CCaMKIIβ as prey or bait (Fig. 3*C*, *lane 4*). These results demonstrate that the N-terminal domains of iPLA$_2$β and CaMKIIβ interact, but the C-terminal domains do not, in agreement with the initial library screening result that the N-terminal domain of CaMKIIβ (residues 34–271) participates in the interaction with iPLA$_2$β. In control experiments, expression of N- or C-terminal fragments of either protein as bait or prey alone resulted in no colonies, as expected (Fig. 3*C*, *lanes 5–8*).

CaMKIIβ Can Be Expressed from Its DNA at High Levels in a Baculovirus-Sf9 Cell System and Retains Activity after Purification

Sf9 cells have been used to express iPLA$_2$β (2, 23, 33), and we found that His-tagged ACaMKIIβ can also be expressed at high levels in Sf9 cells infected with baculovirus containing its cDNA. Cytosol from Sf9 cells infected with baculovirus containing DNA encoding His-tagged ACaMKIIβ was loaded onto TALON metal affinity columns, which were then washed to remove nonadsorbed proteins. Interaction of His-tagged ACaMKIIβ with metal ions on the column resin was then disrupted with imidazole-containing buffers, and this caused desorption of His-tagged ACaMKIIβ protein, which was collected in 0.5-ml fractions of column eluant. Proteins in eluant fractions were analyzed by SDS-PAGE and visualized by immunoblotting using a CaMKII antibody to demonstrate expression and purification of His-tagged ACaMKIIβ (Fig. 4*A*). Purified His-tagged ACaMKIIβ retained catalytic activity reflected by phosphorylation of the synthetic substrate autocamtide-3 in the presence of added Ca^{2+}/CaM. In the absence of added Ca^{2+}/CaM little activity was detected (Fig. 4*B*). The intensity of the immunochemical signal for CaMKIIβ in the eluant fractions (Fig. 4*A*) correlated well with CaMKIIβ activity in these fractions (Fig. 4*B*).

ACaMKIIβ and iPLA$_2$β Interact with Each Other When Co-expressed in Sf9 Cells

To characterize further the interaction between the two proteins, His-tagged ACaMKIIβ and full-length, untagged iPLA$_2$β (hereafter designated “native” iPLA$_2$β) were coexpressed in Sf9 cells to determine whether His-tagged ACaMKIIβ could pull down native iPLA$_2$β from cell cytosol. Sf9 cells were coinfected with baculovirus that contained DNA encoding His-tagged ACaMKIIβ and with baculovirus that contained DNA encoding native iPLA$_2$β. Cytosol was loaded onto TALON metal affinity columns, which were then washed as described above. Imidazole-containing buffer was used to desorb His-tagged CaMKIIβ and any proteins associated with it. Aliquots of eluant fractions were analyzed by SDS-PAGE and immunoblotting with antibodies specific for CaMKIIβ or iPLA$_2$β. His-tagged ACaMKIIβ (Fig. 5*A*, *lower panel*) and native iPLA$_2$β (Fig. 5*A*, *upper panel*) proteins eluted in the same fractions, as detected by immunoblotting. Activity assays for iPLA$_2$β (Fig. 5*B*) and ACaMKIIβ (Fig. 5*C*) indicate that both proteins retain activity after elution. The intensity of the immunochemical signals (Fig. 5*A*) correlated well with the activities of iPLA$_2$β (Fig. 5*B*) and CaMKIIβ (Fig. 5*C*) in the eluant fractions. Similar results were obtained using purified proteins from Sf9 cells (Fig. 6*A*). These findings support the conclusions from yeast two-hybrid assays that these two proteins interact with each other.

The Stoichiometry of the Interaction between iPLA$_2$β and CaMKIIβ

To characterize further the interaction of iPLA$_2$β with ACaMKIIβ, His-tagged ACaMKIIβ was adsorbed onto TALON metal affinity resin, and purified iPLA$_2$β was incubated with the resin. The resin was then washed and loaded into a gravity-flow column, and the interaction between the His tag and the immobilized metal ions was disrupted by elution with imidazole-containing buffer. Proteins in eluant fractions were analyzed by SDS-PAGE and immunoblotting. Fig. 6*A* illustrates that His-tagged ACaMKIIβ (*lower panel*) and iPLA$_2$β (*upper panel*) eluted from the column in the same fractions, which provides additional evidence that these two proteins interact with each other. To determine the molar ratio of the two enzymes in the complex, the dose-response studies illustrated in Fig. 6*B* were performed. The amount of iPLA$_2$β enzyme pulled down by His-tagged ACaMKIIβ increases as the molar ratio increases up to 1:1 but does not increase further at a ratio of 2:1. This suggests that the two enzymes form a complex with 1:1 stoichiometry.

The Calmodulin Antagonist W13 Does Not Prevent the Interaction of CaMKIIβ with iPLA$_2$β

Because both iPLA$_2$β and CaMKIIβ have calmodulin binding domains, calmodulin might mediate the interaction between these two proteins by forming a ternary complex. To evaluate this possibility, the interaction between iPLA$_2$β and CaMKIIβ was examined in the presence and absence of added calmodulin. FLAG-tagged iPLA$_2$β was expressed in Sf9 cells and purified with a FLAG M kit (Sigma). FLAG-tagged iPLA$_2$β was then mixed with TALON metal affinity resin that had previously been loaded with His-tagged ACaMKIIβ in the presence or absence of calmodulin and then washed. When calmodulin was not added, the calmodulin antagonist W13 was added to block binding of any contaminating calmodulin to the target proteins. Adsorbed proteins were eluted from the metal affinity resin with imidazole-containing buffer, and proteins in eluant fractions were analyzed by SDS-PAGE and immunoblotting with iPLA$_2$β-specific antibody. Fig. 7*A* illustrates that added calmodulin is not required for the interaction between iPLA$_2$β and CaMKIIβ and that this interaction is not prevented by the calmodulin antagonist W13. These results are consistent with the findings that the CaM binding site(s) of iPLA$_2$β reside in its C-terminal domain (2) and that the interaction of iPLA$_2$β and CaMKIIβ occurs between their N-terminal domains (Fig. 3*C*).

The Ca^{2+} Chelator EGTA Does Not Prevent the Interaction between iPLA$_2$β and CaMKIIβ

The ability of iPLA$_2$β to bind calmodulin causes iPLA$_2$β preparations purified from cytosol to contain calmodulin, as detected by immunoblotting with calmodulin antibody (data not shown). Previous studies demonstrate that iPLA$_2$β dissociates from calmodulin-agarose in the presence of EGTA (23, 39). To determine the role of calmodulin in the interaction between iPLA$_2$β and CaMKIIβ, we performed an immunoprecipitation study of the interaction of FLAG-tagged iPLA$_2$β with CaMKIIβ in the presence and absence of EGTA. Fig. 7*B* illustrates that in the presence of 10 mM EGTA, FLAG-tagged iPLA$_2$β can still pull down CaMKIIβ from cytosol. The immunoblotting results in Fig. 7*B* illustrate that the amount of CaMKIIβ pulled down by FLAG-tagged iPLA$_2$β is unaffected by EGTA and suggest that calmodulin is not directly involved in the interaction between iPLA$_2$β and CaMKIIβ. In control experiments, the N-terminal FLAG-tagged alkaline phosphatase fusion protein was found not to pull down CaMKIIβ from cytosol, as expected.

The Activities of Both iPLA$_2$β and CaMKIIβ Increase When the Proteins Associate with Each Other

Because results from yeast two-hybrid assays and protein pull-down experiments indicate that the ACaMKIIβ and iPLA$_2$β proteins interact with each other, we next determined whether this interaction affects the catalytic activity of either enzyme. PLA$_2$ activity assays involved measuring radiolabeled free fatty acid release from phospholipid substrates and were performed in buffer supplemented with 10 mM EGTA and 10 mM ATP with no added Ca^{2+}. Under these conditions, adding purified, recombinant, His-tagged ACaMKIIβ to purified, recombinant, His-tagged iPLA$_2$β resulted in a statistically significant increase in PLA$_2$ activity (Fig. 8*A*). Results from dose-response studies under conditions where [iPLA$_2$β] was constant and [CaMKIIβ] was varied indicate that the maximal iPLA$_2$β activity is achieved at a 1:1 molar ratio of the two enzymes (Fig. 8*B*), which is consistent with the finding in Fig. 6*B* that iPLA$_2$β and CaMKIIβ form a complex with 1:1 stoichiometry.

CaMKII activity assays involved measurement of [$^{32}PO_4$] incorporation from [γ-^{32}P]ATP into a model peptide substrate. Fig. 9 illustrates that adding purified, recombinant, His-tagged iPLA$_2$β to purified, recombinant, His-tagged CaMKIIβ resulted in a statistically significant increase in CaMKII activity in the presence of added Ca^{2+}/CaM. Without added Ca^{2+} or CaM, CaMKII activity was low, and it was little affected by adding iPLA$_2$β.

Arachidonic Acid and 2-Lysophosphatidylcholine Inhibit CaMKIIβ Activity

The above results suggest that iPLA$_2$β and CaMKIIβ form a complex and that this affects activities of both enzymes. To examine further the functional relationship between the two enzymes, we measured effects of the iPLA$_2$β reaction products arachidonic acid and 2-lysophosphatidylcholine on CaMKIIβ activity. Fig. 10 illustrates that both arachidonic acid and 2-lysophosphatidylcholine inhibit CaMKIIβ activity in a concentration-dependent manner.

Arachidonic Acid Release from INS-1 Insulinoma Cells Is Suppressed by Inhibitors of CaMKIIβ and iPLA$_2$β

To determine whether evidence for a signaling complex between iPLA$_2$β and CaMKIIβ could be observed in intact β-cells, we examined the effects of the CaMKII inhibitor KN93 and the iPLA$_2$β inhibitor BEL on [^{3}H]arachidonic acid release from prelabeled INS-1 insulinoma cells. Both KN93 and BEL are known to suppress insulin secretion from β-cells (9, 10, 19–22). Fig. 11 illustrates that both the CaMKII inhibitor and the iPLA$_2$β inhibitor suppress [^{3}H]arachidonic acid release from INS-1 cells, which is consistent with an interaction of CaMKIIβ and iPLA$_2$β in β-cells to form a signaling complex.

CaMKIIβ and iPLA$_2$β Form a Complex in Insulin-secreting β Cells

To confirm the formation of an iPLA$_2$β·CaMKIIβ complex in β-cells, we determined whether the two enzymes can be coimmunoprecipitated from INS-1 insulinoma cells. Fig. 12*A* illustrates that both enzymes can be coimmunoprecipitated from parental INS-1 cells and from a stably transfected INS-1 cell line that overexpresses iPLA$_2$β (22) using antibodies against CaMKII (*left panel*). Similar results were obtained in coimmunoprecipitation experiments using antibodies against iPLA$_2$β (*right panel*). This demonstrates the existence of an iPLA$_2$β·CaMKIIβ complex in intact β-cells. Fig. 12*B* illustrates that forskolin, which is an adenylyl cyclase activator that amplifies insulin secretion (22), increases the intensity of the immunochemical signal for iPLA$_2$β that coimmunoprecipitates with CaMKIIβ in INS-1 cells. This suggests that forskolin promotes formation of the iPLA$_2$β·CAMKIIβ complex, and forskolin is also known to induce subcellular redistribution of iPLA$_2$β in INS-1 cells (22).

DISCUSSION

Major PLA$_2$ activities in pancreatic islet β-cells and insulinoma cells are Ca^{2+}-independent, and much evidence indicates that iPLA$_2$β participates in signaling events involved in glucose-induced insulin secretion (19–22, 34, 52). The iPLA$_2$β enzyme is also the predominant PLA$_2$ activity in hippocampus, where it catalyzes arachidonic acid release that is required for long term potentiation (4), which is an electrophysiologic analog of learning. CaMKII is also involved in both insulin secretion (6, 7, 9–11, 45–49, 53) and long term potentiation (54–56). The physiological functions of iPLA$_2$β and CaMKII thus appear to be linked in some cells, such as β-cells and neurons. Another isoform of CaMKII (CaMKIIα) interacts with Group IVA PLA$_2$ (cPLA$_2$) in vascular smooth muscle cells (57), and our findings indicate that CaMKIIβ interacts similarly with iPLA$_2$β to form a complex. Because β-cells express both CaMKIIβ and iPLA$_2$β, a complex of these enzymes could affect β-cell function.

We first observed the complex between iPLA$_2$β and CaMKIIβ by using iPLA$_2$β as bait in yeast two-hybrid screening of a rat brain cDNA library. Formation of a complex between the two enzymes was confirmed in binary yeast two-hybrid assays in which iPLA$_2$β was bait and CaMKIIβ was prey and in the converse assay configuration in which CaMKIIβ was bait and iPLA$_2$β was prey. Pull-down assays with recombinant, His-tagged proteins adsorbed to

metal affinity matrices also provided direct evidence for the physical association of CaMKIIβ and iPLA$_2$β. These findings clearly demonstrate that iPLA$_2$β and CaMKIIβ interact with each other. We have demonstrated here that an immunoprecipitatable complex of these two enzymes exists in insulinoma cells and that the amount of the complex increases upon stimulation of intact β-cells with forskolin, which is an adenylyl cyclase activator that amplifies insulin secretion and induces subcellular redistribution of iPLA$_2$β in β-cells (22).

We have demonstrated previously that depletion of internal Ca^{2+} stores causes activation of iPLA$_2$β in β-cells (23) and in vascular smooth muscle cells (24). It has been demonstrated recently that iPLA$_2$β participates in SOC entry from the extra-cellular space (25, 32), and this process is required for insulin secretion (26–31). Lysophospholipid products of iPLA$_2$β activate SOC channels that mediate capacitative Ca^{2+} influx (25, 32), and CaMKII also affects Ca^{2+} fluxes by potentiating SOC channel activity (58) and regulating T-type voltage-operated calcium channels (59). Our findings indicate that iPLA$_2$β interacts with the specific isoform of CaMKIIβ that is expressed in β-cells and that this interaction affects activities of both iPLA$_2$β and CaMKIIβ. This suggests that CaMKIIβ and iPLA$_2$β form a signaling complex, and this complex represents a potential means to regulate SOC entry.

Such a complex could orchestrate bidirectional signals that result in Ca^{2+} influx into β-cells and insulin secretion. Upon complexation with iPLA$_2$β, CaMKIIβ could displace CaM from iPLA$_2$β (2, 23) and increase iPLA$_2$β activity by relieving tonic inhibition of the enzyme by CaM (2, 8, 23, 24). Lysophospholipids activate SOC channels (32) and are produced by iPLA$_2$ action. Both the CaMKII inhibitor KN93 and the iPLA$_2$β inhibitor BEL inhibit insulin secretion (9, 10, 19–22), and both compounds are also demonstrated here to inhibit arachidonate release from INS-1 insulinoma cells, which supports the possibility that iPLA$_2$β and CaMKIIβ form a signaling complex in β-cells. CaMKIIβ is capable of decoding the frequency of oscillations in intracellular [Ca^{2+}] by its autophosphorylation (54, 61). Autophosphorylated CaMKIIβ has 1,000-fold greater affinity for Ca^{2+}/CaM than does nonphosphorylated CaMKIIβ (62). CaMKIIβ activity is affected by association with iPLA$_2$β (Fig. 9) and by products of iPLA$_2$β action (Fig. 10), including lysophospholipids that also modulate Ca^{2+} channel activities (63, 64). The interaction between CaMKIIβ and iPLA$_2$β at the β-cell plasma membrane could thus affect Ca^{2+} influx and cytosolic [Ca^{2+}], which is a key determinant of insulin secretion (26–31).

Alignment of the deduced amino acid sequences of HCaMKIIβ (48) and ACaMKIIβ, which have been cloned from adult human β-cells and adult rat islets, respectively, reveals more than 99% sequence conservation, and this indicates that there is little species-to-species variation in pancreatic islet β-cell expression of CaMKIIβ isoforms. The expression pattern of CaMKII isoforms does change with development in islets, as reflected by the difference in isoforms expressed in neonatal and adult islets, and there is also tissue-to-tissue heterogeneity in CaMKII isoform expression, as reflected by the different isoforms expressed by islets and brain. The high degree of CaMKIIβ sequence conservation between rat and human islets and the fact that islets express only a single, predominant CaMKIIβ isoform is consistent with the possibility that the islet isoform has a special function in β-cells and that iPLA$_2$β and other proteins that interact with this enzyme modulate that function. It is thus of interest that expression of both iPLA$_2$β and of CaMKIIβ has recently been found to occur at the same stage of differentiation of pancreatic progenitor cells to endocrine progenitor cells during development (60).

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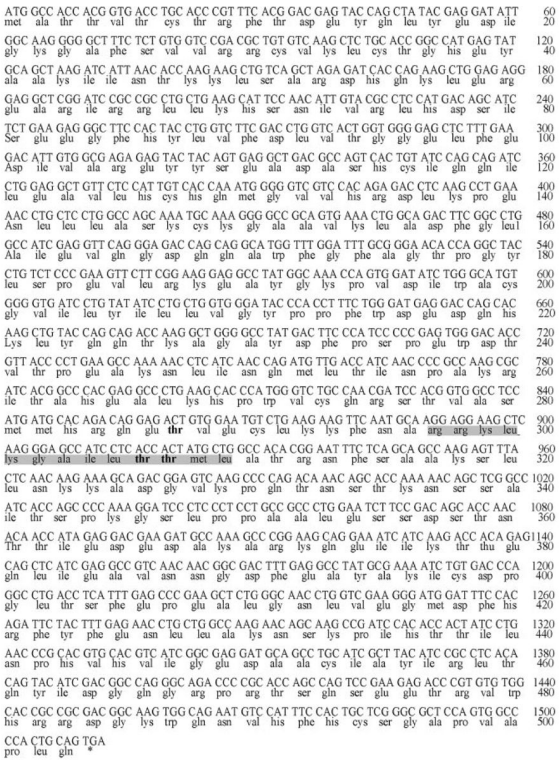

Fig. 1. Nucleotide and deduced amino acid sequences of the CaMKIIβ isoform cloned from an adult rat pancreatic islet cDNA library

The autophosphorylation sites are displayed in *bold type*. The minimal CaM binding sequence is *shaded*.

Fig. 2. Alignment of deduced amino acid sequences of CaMKIIβ isoforms cloned from rat brain (B), adult rat islets (A), adult human β-cells (H), and neonatal rat islets (N)

A compares aligned sequences of CaMKIIβ isoforms from various sources. The variable region is *shaded*. *B* is a graphical alignment of the sequences. The region of difference in amino acid sequence between ACaMKIIβ and HCaMKIIβ is denoted by an *asterisk* (*).

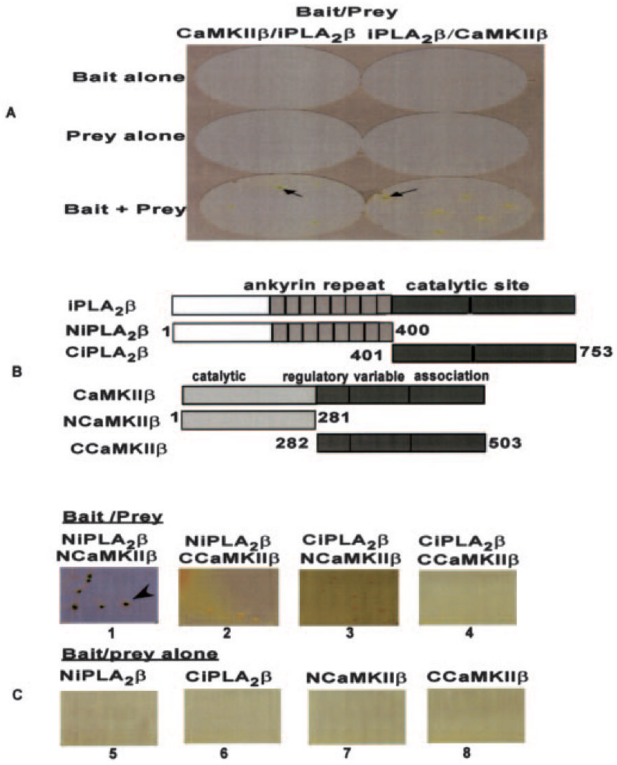

Fig. 3. iPLA$_2$β interacts with CaMKIIβ in yeast cells

A illustrates binary yeast two-hybrid assays performed using full-length iPLA$_2$β (or CaMKIIβ) as bait and full-length CaMKIIβ (or iPLA$_2$β)as prey. *B* contains schematic structures of wild-type iPLA$_2$β and CaMKIIβ and of constructs that correspond to N- or C-terminal fragments of each protein. An iPLA$_2$β N-terminal fragment that contains the ankyrin repeat domain and a C-terminal fragment that contains the catalytic site are shown, as are a CaMKIIβ N-terminal fragment that contains the catalytic domain and a C-terminal fragment that contains the association domain. *C* illustrates binary yeast two-hybrid assays involving coexpression of N- or C-terminal fragments of iPLA$_2$β and of CaMKIIβ as bait/prey pairs together (*lanes 1–4*) or, in control experiments, expression of an N- or C-terminal fragment of one of the proteins alone (*lanes 5–8*). The *blue* colonies reflect specific interactions between two proteins that constitute bait-prey partners in the binary yeast two-hybrid assay. The *arrow* identifies such *blue* colonies formed by the β-galactosidase reaction product after incubation with the chromogenic substrate X-gal.

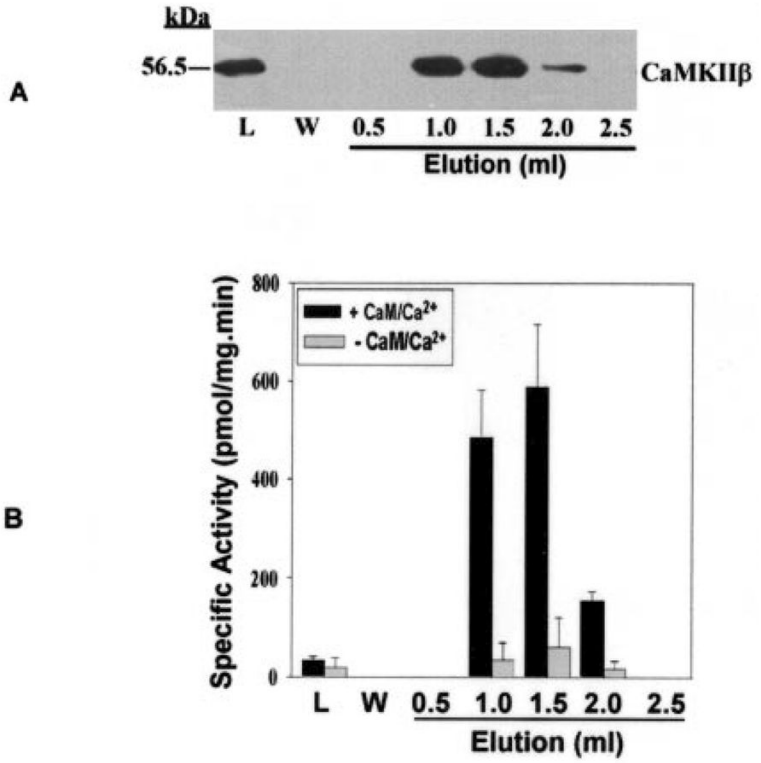

Fig. 4. Expression of His-tagged CaMKIIβ in Sf9 cells and its adsorption to and desorption from metal affinity columns

In *A*, cytosol from Sf9 cells that had been infected with baculovirus containing DNA that encodes His-tagged CaMKIIβ was incubated with TALON metal affinity resin, as described under "Experimental Procedures." The resin was then loaded into a gravity-flow column and washed with buffer, and His-tagged CaMKIIβ was eluted with imidazole-containing buffer and collected in 0.5-ml fractions. Proteins in aliquots of the load (*L*), wash (*W*), and elution fractions were analyzed by SDS-PAGE, and immunoblotting was then performed with CaMKII antibody. In *B*, the protein content of each fraction was measured, and CaMKII activity was determined in the presence (+) or absence (−) of added Ca^{2+}/CaM. When Ca^{2+}/CaM was not added, 1 mM EGTA was added. For each assay, an aliquot of each eluant fraction was mixed with assay buffer, peptide substrate (autocamtide-3), and [γ-^{32}P]ATP, as described under "Experimental Procedures." Displayed values represent the means, and *error bars* denote S.E. (n = 6).

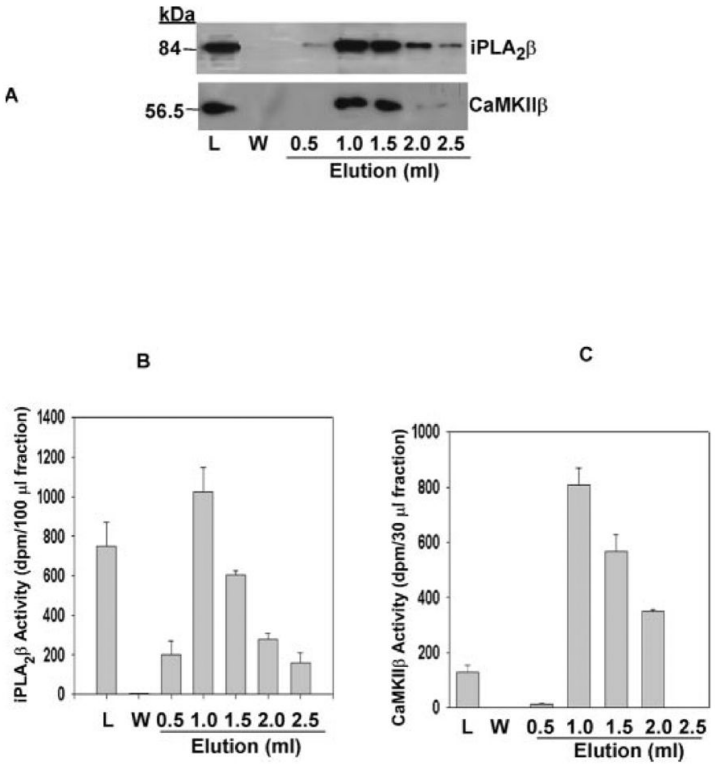

Fig. 5. iPLA$_2$β interacts with CaMKIIβ when the two proteins are coexpressed in Sf9 insect cells

In *A*, Sf9 cells were infected simultaneously with baculovirus containing full-length HisCaMKIIβ and iPLA$_2$β DNAs, cultured, and then homogenized, as described under "Experimental Procedures." Cytosol prepared from homogenates was loaded onto a TALON metal affinity column and washed with buffer. HisCaMKIIβ was eluted with imidazole-containing buffer and collected in 0.5-ml fractions. The proteins in aliquots of load (*L*), wash (*W*), and elution fractions were analyzed by SDS-PAGE, and immunoblotting was performed with antibodies to iPLA$_2$β (*upper panel*) or HisCaMKIIβ (*lower panel*). In *B*, an aliquot of load, wash, or elution fractions was added to assay buffer containing 10 mM EGTA, 1 mM ATP, and 1-palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine substrate. Reactions to measure iPLA$_2$β activity were performed and terminated as described under "Experimental Procedures," and released [^{14}C]linoleic acid was isolated by TLC and measured by liquid scintillation spectrometry. Displayed values represent the means, and *error bars* denote S.E. (n = 6). In *C*, an aliquot of load, wash, or elution fractions was mixed with assay buffer containing 0.1 mM ATP, 0.75 mM $CaCl_2$, 20 μg/ml calmodulin, 20 μM autocamtide-3, and 2 μCi of [γ-^{32}P]ATP and incubated at 30 °C for 3 min to determine CaMKII activity. An aliquot of the reaction mixture was applied to phosphocellulose paper, which was then washed. CaMKII activity was calculated from the amount of phosphorylated autocamtide-3, as determined by liquid scintillation spectrometric measurement of ^{32}P content. Displayed values represent the means, and *error bars* denote S.E. (n = 6).

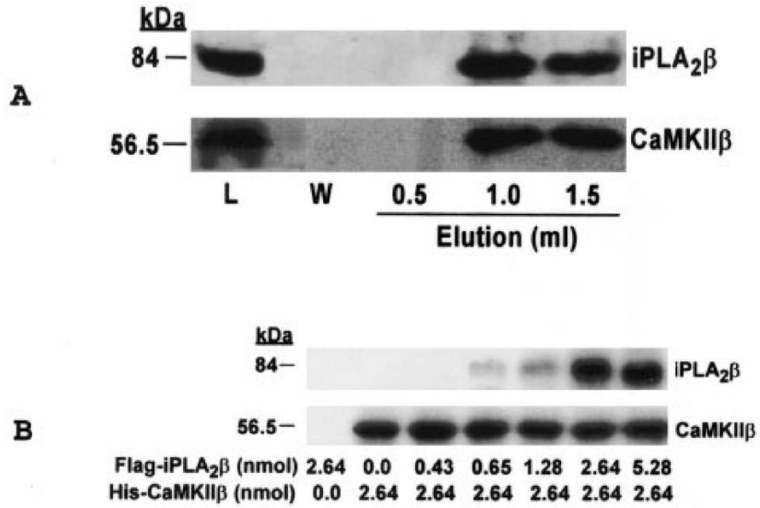

Fig. 6. The stoichiometry of the interaction between iPLA$_2$β and CaMKIIβ

In *A*, purified, recombinant, His-tagged CaMKIIβ from Sf9 cells was mixed with TALON metal affinity resin, and the resin was then washed. Bound CaMKIIβ was measured with a Coomassie protein assay kit. iPLA$_2$β protein expressed in Sf9 cells was purified as described previously (33), and 850 μg (10 nmol) of the protein was mixed with metal affinity resin to which 570 μg (10 nmol) of His-tagged CaMKIIβ had been adsorbed. The mixture was incubated at room temperature for 30 min with shaking, and the resin was washed and loaded onto a gravity-flow column. Bound proteins were eluted with imidazole-containing buffer and collected in 0.5-ml fractions. Proteins in aliquots of the load (*L*), wash (*W*), and elution fractions were analyzed by 10% SDS-PAGE, and immunoblotting was then performed with antibodies specific for iPLA$_2$β (*upper panel*) or CaMKIIβ (*lower panel*). In *B*, 200 μl of metal affinity resin slurry to which 150 μg (2.64 nmol) of His-tagged CaMKIIβ had been adsorbed was mixed with FLAG-tagged iPLA$_2$β in amounts that varied from 0 to 5.28 nmol. The mixture was incubated at 4 °C overnight with shaking, and the resin was then washed to remove noncomplexed proteins. Proteins were eluted from the metal affinity resin and analyzed by 10% SDS-PAGE. Immunoblotting was then performed with primary antibodies specific for iPLA$_2$β (*upper panel*) or CaMKIIβ (*lower panel*).

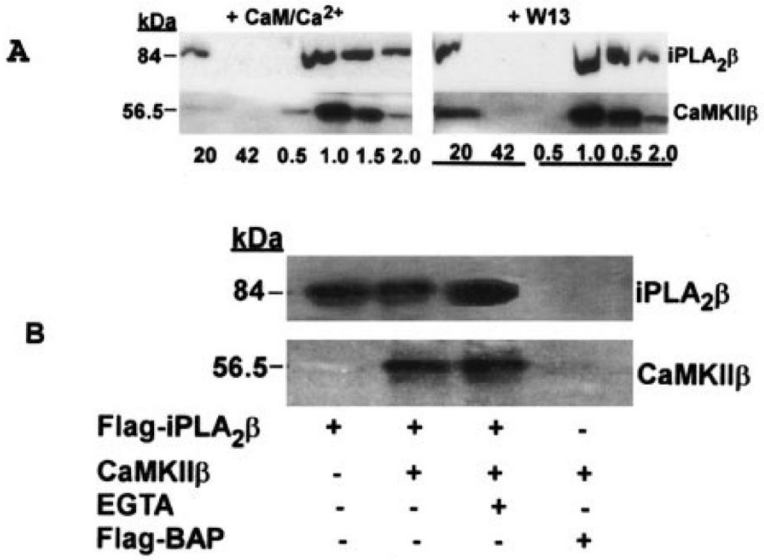

Fig. 7. The interaction between iPLA$_2$β and CaMKIIβ does not require added calmodulin and is not prevented by a calmodulin antagonist or the Ca^{2+} chelator EGTA

In *A*, FLAG-tagged iPLA$_2$β expressed in Sf9 cells was purified with a FLAG M kit. Recombinant, purified, His-tagged CaMKIIβ was adsorbed to TALON metal affinity resin. The FLAG-tagged iPLA$_2$β was incubated with the resin to which His-tagged CaMKIIβ had been adsorbed at room temperature for 30 min with shaking in the presence of 0.25 mM Ca^{2+} and 1 mM CaM (*upper left panel*) or 0.4 mM calmodulin antagonist W13 (*upper right panel*). The resin was then washed, and adsorbed proteins were eluted with imidazole-containing buffer. Aliquots of wash and elution fractions were analyzed by SDS-PAGE and immunoblotting with antibody specific for iPLA$_2$β or CaMKII. In *B*, cytosol was prepared from baculovirus-infected Sf9 cells that expressed FLAG-iPLA$_2$β, CaMKIIβ without a FLAG tag, or the control fusion protein N-terminal FLAG-tagged alkaline phosphatase (*Flag-BAP*). Binary mixtures of cytosols were prepared and incubated with anti-FLAG M2 affinity resin for 2 h at 4 °C in the presence or absence of 10 mM EGTA. Immunoprecipitated material was recovered by centrifugation and washed four times with wash buffer. Samples immunoprecipitated with anti-FLAG affinity resin were eluted with buffer containing FLAG peptide. Proteins in the eluant were analyzed by 10% SDS-PAGE and transferred onto a nylon membrane, and immunoblotting was performed with iPLA$_2$β or CaMKII antibodies.

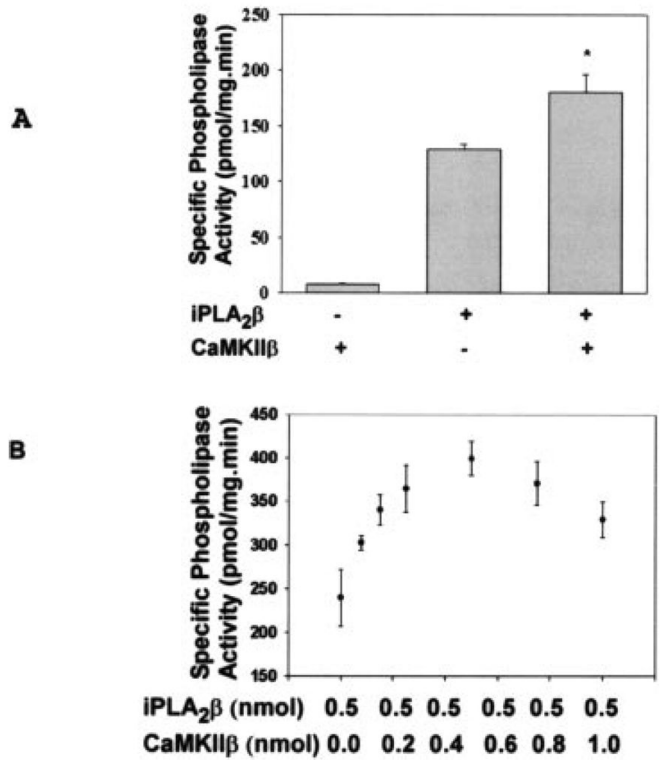

Fig. 8. Influence of CaMKIIβ on iPLA$_2$β activity

In *A*, His-tagged CaMKIIβ and His-tagged iPLA$_2$β were purified with TALON metal affinity columns. Purified, His-tagged CaMKIIβ, His-tagged iPLA$_2$β, or both were then added to buffer containing 10 mM ATP, 10 mM EGTA, and the radiolabeled substrate 1-palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine. The iPLA$_2$ activity was then calculated from released [^{14}C]linoleate as in Fig. 5. Values are represented as the mean ± S.E. (n = 4). Statistical significance is denoted by an *asterisk* (*), which indicates a p value < 0.05. In *B*, iPLA$_2$β activity assays were performed in the presence of 0.5 nmol of iPLA$_2$β and the indicated amounts of CaMKIIβ. Displayed values represent the means ± S.E. (n = 3).

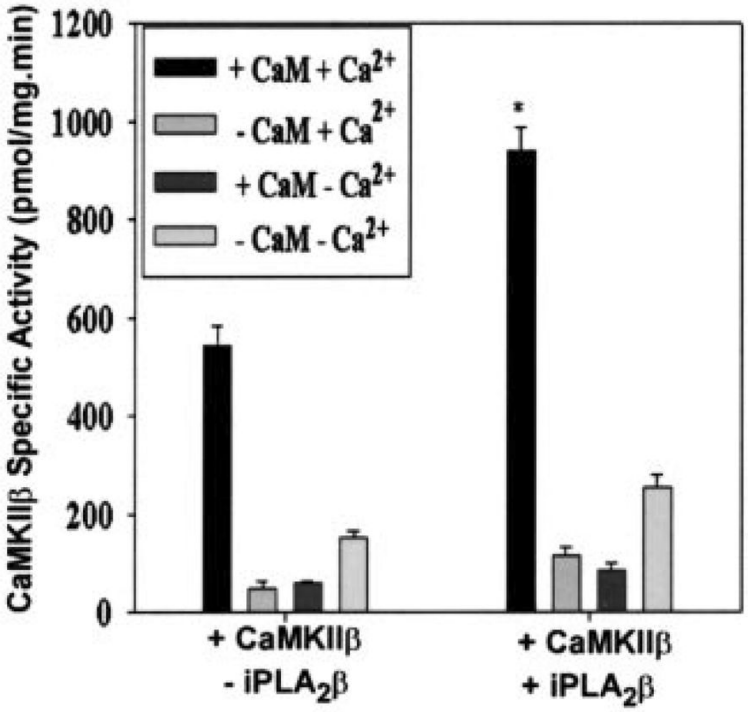

Fig. 9. Influence of iPLA$_2$β on CaMKIIβ activity

CaMKIIβ activity was measured in the presence (+) or absence (−) of iPLA$_2$β, Ca^{2+}, and CaM. For each assay, His-tagged CaMKIIβ was mixed with assay buffer containing ATP, autocamtide-3 substrate, and [γ-^{32}P]ATP. CaMKII activity was calculated from the amount of phosphorylated autocamtide-2 as in Fig. 4. Values are represented as the mean ± S.E. (n = 5). Statistical significance is denoted by an *asterisk*, which indicates a p value < 0.01 compared with the group to which no iPLA$_2$β was added (−iPLA$_2$β).

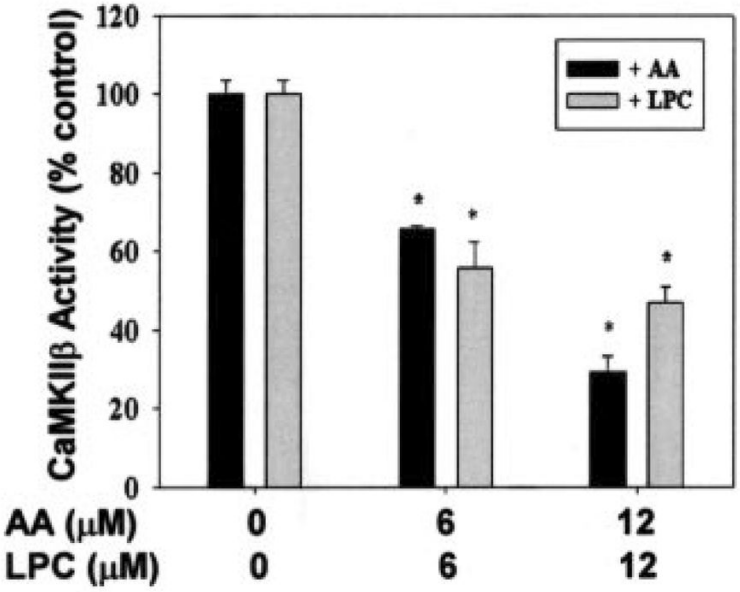

Fig. 10. Inhibition of CaMKIIβ activity by arachidonic acid (*AA*) and lysophosphatidylcholine (*LPC*)

CaMKIIβ activity was measured in the presence or absence of arachidonic acid or lysophosphatidylcholine as in Fig. 4. For each assay, two separate measurements were performed simultaneously, one in the presence and the other in the absence of added Ca^{2+}/CaM. Activity values were calculated from the difference between these two measurements and are represented as the mean ± S.E. ($n = 3$). Statistical significance is denoted by an *asterisk* (*), which indicates a p value < 0.05 compared with control.

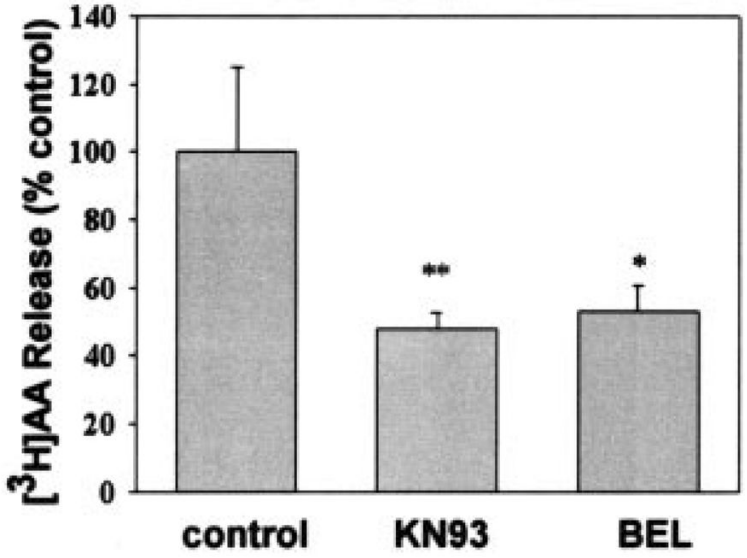

Fig. 11. Arachidonic acid (*AA*) release from INS-1 cells is suppressed by inhibitors of iPLA$_2$β and CaMKIIβ

INS-1 cells were prelabeled with [^{3}H]arachidonic acid and then washed free of unincorporated radiolabel. The labeled cells were then treated without or with 20 μM BEL or 8 μM KN93. [^{3}H]Arachidonic acid release was then measured as described under "Experimental Procedures." Release values are represented as the mean ± S.E. (n = 3). Statistical significance is denoted by an *asterisk* (*) or a *double asterisk* (**), which indicates a p value < 0.05 or 0.01, respectively, compared with control.

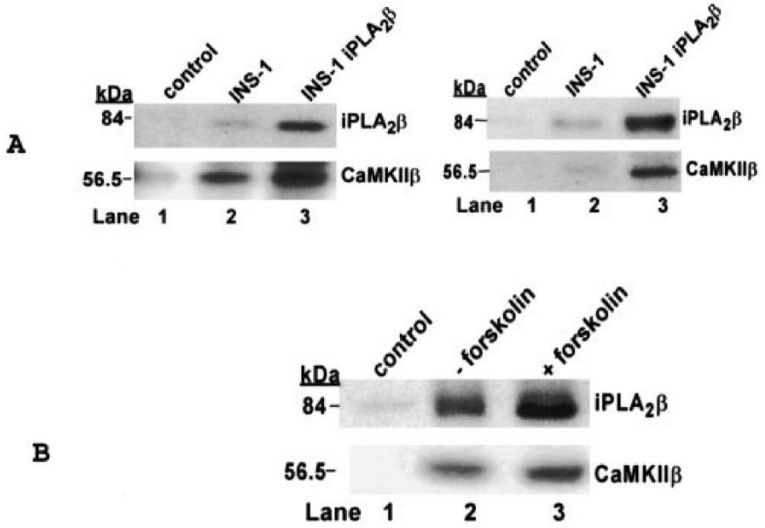

Fig. 12. Forskolin stimulates complex formation between iPLA$_2$β and CaMKIIβ in INS-1 insulinoma cells

A illustrates coimmunoprecipitation of iPLA$_2$β and CaMKIIβ. In *lane 1* of the *left panel*, control preimmune serum was used for sham immunoprecipitation of INS-1 cell cytosol as a negative control. In *lanes 2* and *3* of the *left panel*, cytosol from INS-1 cells (*lane 2*) or from INS-1 cells that overexpress iPLA$_2$β (*lane 3*) were incubated with anti-CaMKIIβ antibody attached to protein A-agarose. The immunoprecipitate was collected by centrifugation, washed, boiled in SDS-PAGE sample loading buffer, and analyzed by SDS-PAGE. After transfer of proteins to nylon membranes, immunoblotting was performed with antibodies against iPLA$_2$β (*upper blot*) or CaMKIIβ (*lower blot*). Similar results were obtained from the reverse immunoprecipitation experiment (*right panel* of *A*), in which cytosol from INS-1 cells (*lane 2*) or INS-1 cells that overexpress iPLA$_2$β (*lane 3*) was immunoprecipitated with iPLA$_2$β antibody-protein A-agarose. In *lane 1* of *B*, control preimmune serum was used in sham immunoprecipitation of INS-1 cell cytosol as a negative control. In *lanes 2* and *3* of *B*, INS-1 cells that overexpress iPLA$_2$β were incubated without (*lane 2*) or with (*lane 3*) 4 μM forskolin. The cytosol was then immunoprecipitated with CaMKIIβ antibody-protein A-agarose. After SDS-PAGE analyses of the immunoprecipitates, immunoblotting was performed with iPLA$_2$β antibody (*upper blot*) or with CaMKIIβ antibody (*lower blot*).