Group VIA Phospholipase A₂ Forms a Signaling Complex with the Calcium/Calmodulin-dependent Protein Kinase IIβ Expressed in Pancreatic Islet β-Cells*

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Insulin-secreting pancreatic islet β-cells express a Group VIA Ca²⁺-independent phospholipase A₂ (iPLA₂) that contains a calmodulin binding site and protein interaction domains. We identified Ca²⁺/calmodulin-dependent protein kinase IIβ (CaMKIIβ) as a potential iPLA₂β-interacting protein by yeast two-hybrid screening of a cDNA library using iPLA₂β 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 iPLA₂β DNA as prey confirmed interaction of the enzymes, as did assays with CaMKIIβ as prey and iPLA₂β DNA as bait. His-tagged CaMKIIβ immobilized on metal affinity matrices bound iPLA₂β, and this did not require exogenous calmodulin and was not prevented by a calmodulin antagonist or the Ca²⁺-chelator EGTA. Activities of both enzymes increased upon their association, and iPLA₂β reaction products reduced CaMKIIβ activity. Both the iPLA₂β inhibitor bromoeno lactone and the CaMKIIβ inhibitor KN93 reduced arachidonate release from INS-1 insulinoma cells, and both inhibit insulin secretion. CaMKIIβ and iPLA₂β can be coimmunoprecipitated from INS-1 cells, and forskolin, which amplifies glucose-induced insulin secretion, increases the abundance of the immuno precipitable complex. These findings suggest that iPLA₂β 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 A₂ (PLA₂)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). This Group VIA PLA₂ designated iPLA₂β has a molecular mass of 84–88 kDa and does not require Ca²⁺ for catalysis (2). Various splice variants of iPLA₂β 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 n-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 [Ca²⁺] (2), and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is an important β-cell [Ca²⁺] sensor and mediator of Ca²⁺-dependent events in insulin secretion (6–11). Much evidence (12–22) suggests that iPLA₂β also participates in insulin secretion, including the facts that the mechanism-based bromoeno lactone (BEL) inhibitor of iPLA₂β suppresses glucose-induced hydrolysis of arachidonate from islet membrane phospholipids, the rise in β-cell cytosolic [Ca²⁺], and insulin secretion (19–22).

Depleting intracellular Ca²⁺ stores activates iPLA₂β in β-cells and vascular smooth muscle cells (23, 24), and iPLA₂β participates in store-operated entry of Ca²⁺ 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 Ca²⁺ stores and Ca²⁺-cellular Ca²⁺ and calmodulin participate in cross-talk between Ca²⁺ stores and SOC channels (25, 32). Lipid signaling molecules (e.g. lysophospholipids) and Ca²⁺-sensitive kinases and phosphatases (e.g. CaMKIIβ and calcineurin) are also proposed to affect these interactions (9, 10, 25, 32). Mechanisms whereby iPLA₂β participates in glucose-induced rises in β-cell cytosolic [Ca²⁺] and insulin secretion are likely to involve Ca²⁺-sensitive regulation of modulatory and effector proteins by phosphorylation-dephosphorylation events (9, 10), and iPLA₂β activity is also affected by local [Ca²⁺] increments that relieve its tonic inhibition by Ca²⁺/calmodulin (2, 8, 25).

The amino acid sequence of iPLA₂β 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-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one; CaM, calmodulin; CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; iPLA₂, calcium-independent phospholipase A₂; Pipes, 1,4-piperazinediethanesulfonic acid; SOC, store-operated calcium channel; X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside.
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\(^{2+}\)-dependent processes in subcellular zones involved in Ca\(^{2+}\) signaling. CaMKII is an important Ca\(^{2+}\)-signaling effector and serves as a gate that temporarily 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\(_2\) interact functionally (2, 8, 23, 24, 33), and the iPLA\(_2\)-β domain from residues 650–722 contains a calmodulin binding site (2).

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\(_2\)-β to understand better its role in signaling, we performed yeast two-hybrid screening and have found that iPLA\(_2\)-β 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\(_2\) and CaMKIIβ activities, thereby defining a signaling complex.

**EXPERIMENTAL PROCEDURES**

**Materials**—The materials (γ-32P)ATP, 55 mCi/μmol (16:0/[14C]18:2)-glycerophosphocholine-[1-palmitoyl-2-[14C]linoleoyl-sn-glycerol-3-phosphocholine, rainbow molecular mass standards, enhanced chemiluminescence (ECL) reagent, and [3H]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, kaempferol, amphotericin B, and heat-inactivated calf serum were obtained from Sigma. T4 DNA ligase was obtained from Promega. The iPLA\(_2\) and CaMKII were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The calmodulin inhibitor W13 was obtained from Calbiochem. The iPLA\(_2\)-β 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 to screen the library. The fidelity of constructs was confirmed, and the interaction involved in 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).

**Interaction of iPLA\(_2\)-β with CaMKIIβ**

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 genomic DNA sequences that are conserved in the mouse and rat brain CaMKIIβ DNA sequences (sense, 5′-ATCGCCACCG-CATGGCCACC-3′; antisense, 5′-CAGGGCGCAGCTTCACTGGAC-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 cDNA 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, screened as described above, and 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\(_2\), 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. DNA containing the entire coding sequence of His-tagged CaMKIIβ, His-tagged iPLA\(_2\)-β, or FLAG-tagged iPLA\(_2\)-β was cloned into the Sall-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 plasmids were verified by 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 CaMKIIβ. 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**—For these 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 (2 h, 4 °C, gentle rotation) in the presence or absence of Ca\(^{2+}\) to determine the metal affinity of the CaMKIIβ interactome. The iPLA\(_2\)-β and CaMKIIβ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The calmodulin inhibitor W13 was obtained from Calbiochem. The iPLA\(_2\)-β 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\(_2\)-β cDNA cloned from rat pancreatic islets was ligated into the Sall-EcoRI sites of the two-hybrid BD pAS2-1 vector, which contained a TRP1 gene for selection, resulting in in-frame fusion of iPLA\(_2\)-β 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. Luck of autonomous activation by the iPLA\(_2\)-β 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, and color development was scored by centrifugation (15,000 × g, 20 min) and incubated with 100 μl of anti-FLAG M2 antibody (2 h, 4 °C, gentle rotation) in the presence or absence of 20 μM α-CHERG7 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...
Interaction of iPLA\(_{2}\beta\) with CaMKII\(\beta\)

Cloning CaMKII\(\beta\) from a Rat Islet cDNA Library Reveals Tissue Specificity and a Developmental Profile of CaMKII\(\beta\) Isoform Expression—Pancreatic islets express distinct CaMKII isoforms, and adult rat islets express predominantly the CaMKII\(\beta\) isoform (48, 49). To determine whether CaMKII isoform(s) were expressed in rat islets, like those in rat brain, we interact with iPLA\(_{2}\beta\), we cloned CaMKII\(\beta\) 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 CaMKII\(\beta\). 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 CaMKII\(\beta\) isoform cloned from adult rat islets (ACaMKII\(\beta\)). Fig. 2A demonstrates sequence alignments for CaMKII\(\beta\) from rat brain (BCaMKII\(\beta\)), adult rat islets (ACaMKII\(\beta\)), human \(\beta\)-cells (HCaMKII\(\beta\)), and neonatal rat islets (NCaMKII\(\beta\)).

The CaMKII\(\beta\) 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 CaMKII\(\beta\) isoform is distinct from previously (50, 51) described rat isoforms. Analysis of nucleotide sequences revealed that ACaMKII\(\beta\) differs from BCaMKII\(\beta\) (50) by the lack of sequence corresponding to the first (residues 316–339) and second (residues 392–408) variable domains (Fig. 2A). ACaMKII\(\beta\) differs from NCaMKII\(\beta\) (51) by the absence of the sequence from residues 370 to 456 in the association domain (Fig. 2B). Sequence alignments revealed 99.4% amino acid sequence identity between ACaMKII\(\beta\) and the HCaMKII\(\beta\) isoform cloned from human insulinoma cells (48). The ACaMKII\(\beta\) and HCaMKII\(\beta\) sequences differ only in 3 amino acid residues in variable domain 2 (Fig. 2B).

To search for other subtypes of CaMKII\(\beta\) 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 ACaMKII\(\beta\) sequence, but we observed no other CaMKII\(\beta\) subtype in adult rat islets (not shown). Adult rat pancreatic islets and adult human \(\beta\)-cells thus express mRNA that encodes a CaMKII\(\beta\) 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 CaMKII\(\beta\) isoform expression, but there is little rat-to-human species heterogeneity in the CaMKII\(\beta\) isoform expressed in adult pancreatic islet \(\beta\)-cells.

Binary Yeast Two-hybrid Assays Confirm the Interaction between iPLA\(_{2}\beta\) and CaMKII\(\beta\)—To confirm the interaction between iPLA\(_{2}\beta\) and ACaMKII\(\beta\) observed in yeast two-hybrid screening experiments, binary yeast two-hybrid assays were performed. We first used ACaMKII\(\beta\) as bait and iPLA\(_{2}\beta\) as prey. When prey or 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. 3B, left column) that reflect interaction between ACaMKII\(\beta\) and iPLA\(_{2}\beta\). When the bait and prey DNA were switched (so that iPLA\(_{2}\beta\) was bait, and ACaMKII\(\beta\) was prey), similar results were obtained (Fig. 3B, right column). These results reflect a specific interaction between iPLA\(_{2}\beta\) and CaMKII\(\beta\).

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\(_{2}\beta\) as the bait or prey and N- or C-terminal fragments of CaMKII\(\beta\) as the prey or bait. Fig. 3B
shows the schematic representation of wild-type iPLA₂β and CaMKIIβ proteins and of their N- and C-terminal fragments. When the N-terminal fragment of iPLA₂β (NiPLA₂β) was used as the bait or prey and the N-terminal fragment of CaMKII (NCaMKII) 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. 3C, lane 1). When an N-terminal fragment (NiPLA₂β or NCaMKII) was used as bait or prey and a C-terminal fragment (CiPLA₂β or CCaMKII) as the prey or bait, only small colonies formed after incubation at 30 °C for 4 days (Fig. 3C, 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 iPLA₂β and CaMKIIβ are weak and nonspecific. No colonies formed when the C-terminal fragment CiPLA₂β was used as bait or prey and CCaMKII as prey or bait (Fig. 3C, lane 4). These results demonstrate that the N-terminal domains of iPLA₂β 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 iPLA₂β. 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. 3C, 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 iPLA₂β (2, 23, 33), and we found that His-tagged ACaMKII 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 ACaMKII was loaded onto TALON metal affinity columns, which were then washed to remove nonadsorbed proteins. Interaction of His-tagged ACaMKII with metal ions on the column resin was then disrupted with imidazole-containing buffers, and this caused desorption of His-tagged ACaMKII 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 ACaMKII (Fig. 4A). Purified His-tagged ACaMKII retained catalytic activity reflected by phosphorylation of the synthetic substrate autocamtide-3 in the presence of added Ca²⁺/CaM. In the absence of added Ca²⁺/CaM little activity was detected (Fig. 4B). The intensity of
the immunochemical signal for CaMKIIβ in the eluant fractions (Fig. 4A) correlated well with CaMKIIβ activity in these fractions (Fig. 4B). The Stoichiometry of the Interaction between iPLA2β and CaMKIIβ—To characterize further the interaction of iPLA2β with CaMKIIβ, His-tagged iPLA2β (Fig. 5A, upper panel) and native iPLA2β (Fig. 5A, lower panel) proteins eluted in the same fractions, as detected by immunoblotting. Activity assays for iPLA2β (Fig. 5B) and CaMKIIβ (Fig. 5C) indicate that both proteins retain activity after elution. The intensity of the immunochemical signals (Fig. 5A) correlated well with the activities of iPLA2β (Fig. 5B) and CaMKIIβ (Fig. 5C) in the eluant fractions. Similar results were obtained using purified proteins from S9 cells (Fig. 6A). These findings support the conclusions from yeast two-hybrid assays that these two proteins interact with each other.
were analyzed by SDS-PAGE and immunoblotting. Fig. 6A illustrates that His-tagged ACaMKIIβ (lower panel) and iPLA2β (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. 6B were performed. The amount of iPLA2β 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 iPLA2β—Because both iPLA2β 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 iPLA2β and CaMKIIβ was examined in the presence and absence of added calmodulin. FLAG-tagged iPLA2β was expressed in Sf9 cells and purified with a FLAG M kit (Sigma). FLAG-tagged iPLA2β 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 eluent fractions were analyzed by SDS-PAGE and immunoblotting with iPLA2β-specific antibody. Fig. 7A illustrates that added calmodulin is not required for the interaction between iPLA2β 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 iPLA2β reside in its C-terminal domain (2) and that the interaction of iPLA2β and CaMKIIβ occurs between their N-terminal domains (Fig. 3C).

The Ca2⁺ Chelator EGTA Does Not Prevent the Interaction between iPLA2β and CaMKIIβ—The ability of iPLA2β to bind calmodulin causes iPLA2β preparations purified from cytols to contain calmodulin, as detected by immunoblotting with calmodulin antibody (data not shown). Previous studies demonstrate that iPLA2β dissociates from calmodulin-agarose in the presence of EGTA (23, 39). To determine the role of calmodulin in the interaction between iPLA2β and CaMKIIβ, we performed an immunoprecipitation study of the interaction of FLAG-tagged iPLA2β with CaMKIIβ in the presence and absence of EGTA. Fig. 7B illustrates that in the presence of 10 mM EGTA, FLAG-tagged iPLA2β can still pull down CaMKIIβ from cytols. The immunoblotting results in Fig. 7B illustrate that the amount of CaMKIIβ pulled down by FLAG-tagged iPLA2β is unaffected by EGTA and suggest that calmodulin is not directly involved in the interaction between iPLA2β 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 iPLA2β 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 iPLA2β proteins interact with each other, we next determined whether this interaction affects the catalytic activity of either enzyme. PL2 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 Ca2+.

Under these conditions, adding purified, recombinant, His-tagged ACaMKIIβ to purified, recombinant, His-tagged iPLA2β resulted in a statistically significant increase in PL2 activity (Fig. 8A). Results from dose-response studies under conditions where [iPLA2β] was constant and [CaMKIIβ] was varied indicate that the maximal iPLA2β activity is achieved at a 1:1 molar ratio of the two enzymes (Fig. 8B), which is consistent with the finding in Fig. 6B that iPLA2β and CaMKIIβ form a complex with 1:1 stoichiometry.

CaMKII activity assays involved measurement of [32P] incorporation from [γ-32P]ATP into a model peptide substrate. Fig. 9 illustrates that adding purified, recombinant, His-tagged iPLA2β to purified, recombinant, His-tagged CaMKIIβ resulted in a statistically significant increase in CaMKII activity in the presence of added Ca2+/CaM. Without added Ca2+ or CaM, CaMKIIβ activity was low, and it was little affected by adding iPLA2β.

Arachidonic Acid and 2-Lyso phosphatidylcholine Inhibit CaMKIIβ Activity—The above results suggest that iPLA2β 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 iPLA2β reaction products arachidonic acid and 2-lyso phosphatidylcholine on CaMKIIβ activity. Fig. 10 illustrates that both arachidonic acid and 2-lyso phosphatidylcholine inhibit CaMKIIβ activity in a concentration-dependent manner.

Arachidonic Acid Release from INS-1 Insulinoma Cells Is Suppressed by Inhibitors of CaMKIIβ and iPLA2β—To determine whether evidence for a signaling complex between iPLA2β and CaMKIIβ could be observed in intact β-cells, we examined the effects of the CaMKII inhibitor KN93 and the iPLA2β inhibitor BEL on [3H]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 iPLA2β inhibitor suppress [3H]arachidonic acid release from INS-1 cells, which is consistent with an interaction of CaMKIIβ and iPLA2β in β-cells to form a signaling complex.

CaMKIIβ and iPLA2β Form a Complex in Insulin-secreting β Cells—To confirm the formation of an iPLA2β-CaMKIIβ complex in β-cells, we determined whether the two enzymes can be immunoprecipitated from INS-1 insulinoma cells. Fig. 12A illustrates that both enzymes can be immunoprecipitated from parental INS-1 cells and from a stably transfected INS-1 cell line that overexpresses iPLA2β (22) using antibodies against CaMKII (left panel). Similar results were obtained in immunoprecipitation experiments using antibodies against iPLA2β (right panel). This demonstrates the existence of an iPLA2β-CaMKIIβ complex in intact β-cells. Fig. 12B illustrates that forskolin, which is an adenyl cyclase activator that amplifies insulin secretion (22), increases the intensity of the
immunoochemical signal for iPLA$_2$ that coimmunoprecipitates with CaMKII in INS-1 cells. This suggests that forskolin promotes formation of the iPLA$_2$-CaMKII complex, and forskolin is also known to induce subcellular redistribution of iPLA$_2$ in INS-1 cells (22).

**Discussion**

Major PLA$_2$ activities in pancreatic islet $\beta$-cells and insulinoma cells are Ca$^{2+}$-independent, and much evidence indicates that iPLA$_2$ participates in signaling events involved in glucose-induced insulin secretion (19–22, 34, 52). The iPLA$_2$ 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$_2$ and CaMKII thus appear to be linked in some cells, such as $\beta$-cells and neurons. Another isoform of CaMKII (CaMKII$\alpha$) interacts with Group IVA PLA$_2$ (cPLA$_2$) in vascular smooth muscle cells (57), and our findings indicate that CaMKII$\beta$ interacts similarly with iPLA$_2$ to form a complex. Because $\beta$-cells express both CaMKII$\beta$ and iPLA$_2$, a complex of these enzymes could affect $\beta$-cell function.

We first observed the complex between iPLA$_2$ and CaMKII by using iPLA$_2$ 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$_2$ was bait and CaMKII was prey and in the converse assay configuration in which CaMKII was bait and iPLA$_2$ was prey. Pull-down assays with recombinant, His-tagged proteins adsorbed to metal affinity columns. Purified, His-tagged CaMKII, His-tagged iPLA$_2$, 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 $\pm$ S.E. ($n = 4$). Statistical significance is denoted by an asterisk (*), which indicates a $p$ value < 0.05. Statistical significance is denoted by an asterisk (**), which indicates a $p$ value < 0.01, respectively, compared with control.
CaMKII activity is affected by association with iPLA2β (Fig. 9) and by products of iPLA2β action (Fig. 10), including lysosphopholipids that also modulate Ca2+ channel activities (63, 64). The interaction between CaMKIIβ and iPLA2β at the β-cell plasma membrane could thus affect Ca2+ influx and cytosolic [Ca2+]i, 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 iPLA2β and other proteins that interact with this enzyme modulate that function. It is thus of interest that expression of both iPLA2β 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. 12. Forskolin stimulates complex formation between iPLA2β and CaMKIIβ in INS-1 insulinoma cells. A illustrates co-immunoprecipitation of iPLA2β 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 iPLA2β (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 iPLA2β (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 iPLA2β (lane 3) was immunoprecipitated with iPLA2β 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 iPLA2β 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 antibodies against iPLA2β (upper blot) or with CaMKIIβ antibody (lower blot).

redistribution of iPLA2β in β-cells (22).

We have demonstrated previously that depletion of internal Ca2+ stores causes activation of iPLA2β in β-cells (23) and in vascular smooth muscle cells (24). It has been demonstrated recently that iPLA2β participates in SOC entry from the extracellular space (25, 32), and this process is required for insulin secretion (26–31). Lysosphopholipid products of iPLA2β activate SOC channels that mediate capacitative Ca2+ influx (25, 32), and CaMKII also affects Ca2+ fluxes by potentiating SOC channel activity (58) and regulating T-type voltage-operated calcium channels (59). Our findings indicate that iPLA2β interacts with the specific isoform of CaMKIIβ that is expressed in β-cells and that this interaction affects activities of both iPLA2β and CaMKIIβ. This suggests that CaMKIIβ and iPLA2β 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 Ca2+ influx into β-cells and insulin secretion. Upon complexification with iPLA2β, CaMKIIβ could displace CaM from iPLA2β (2, 23) and increase iPLA2β activity by relieving tonic inhibition of the enzyme by CaM (2, 8, 23, 24). Lysosphopholipids activate SOC channels (32) and are produced by iPLA2β action. Both the CaMKIIβ inhibitor KN93 and the iPLA2β 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 iPLA2β and CaMKIIβ form a signaling complex in β-cells. CaMKIIβ is capable of decoding the frequency of oscillations in intracellular [Ca2+]i by its autophosphorylation (54, 61). Autophosphorylated CaMKIIβ has 1,000-fold greater affinity for Ca2+/CaM than does nonphosphorylated CaMKIIβ (62).
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