0013-7227/01/$03.00/0
Endocrinology

Vol. 142, No. 3
*Printed in U.S.A.*

# Targeted Protein Kinase A and PP-2B Regulate Insulin Secretion through Reversible Phosphorylation*

LINDA B. LESTER, MAREE C. FAUX, J. BRIAN NAUERT, AND JOHN D. SCOTT

*Division of Endocrinology (L.B.L., J.B.N.), Oregon Health Sciences University, Portland, Oregon 97201; Ludwig Institute (M.C.F.), Melbourne, Australia; Howard Hughes Medical Institute (J.D.S.), Vollum Institute, Portland, Oregon 97201*

**ABSTRACT**

Protein kinases and phosphatases play key roles in integrating signals from various insulin secretagogues. In this study, we show that the activities of the cAMP-dependent protein kinase (PKA) and the calcium/calmodulin-dependent phosphatase, PP-2B are coordinated resulting in the regulation of insulin secretion. Transient inhibition of PP-2B, using the immunosuppressant FK506, increased forskolin stimulated insulin secretion by 2.5-fold ± 0.3 (n = 6) in rat islets and RINm5F cells. Surprisingly, forskolin treatment resulted in the dephosphorylation of the vesicle-associated protein synapsin 1 and increased PP-2B activity by 2.98 ± 0.97-fold (n = 4). One potential explanation for the observed coordination of PKA and PP-2B activity is their colocalization through a mutual anchoring protein, AKAP79/150. Accordingly, RINm5F cells expressing AKAP79 exhibited decreased insulin secretion, reduced PP-2B activity and were insensitive to FK506. This suggests that AKAP targeting of PKA and PP-2B maintains a signal transduction complex that may regulate reversible phosphorylation events involved in insulin secretion. (*Endocrinology* **142:** 1218–1227, 2001)

INSULIN secretion from pancreatic β cells is regulated by the intracellular metabolism of nutrients, primarily glucose (1). However, nonnutrient signals can augment this process. For example, the hormone glucagon-like peptide 1 (GLP-1) is a potent insulin secretagogue but only in the presence of elevated glucose levels (2–7). These observations suggest that nutrient and nonnutrient signaling pathways are integrated in the regulation of insulin secretion. One mechanism integrating these signals is the activation of specific kinases and phosphatases resulting in the reversible phosphorylation of β cell proteins (8–11). This concept is supported by a recent study where activation of kinases, including protein kinase C (PKC), protein kinase A (PKA), and the calcium, calmodulin-dependent kinase (CaM K II) and concomitant inhibition of protein phosphatases lead to enhanced insulin secretion in β cells (12). Furthermore, transient inhibition of the calcium, calmodulin-dependent phosphatase, PP-2B, by the immunosuppressant drug cyclosporin (CsA) increased insulin secretion (13). This finding contrasts a body of evidence suggesting that long-term treatment with the immunosuppressants CsA or FK506 impairs insulin secretion (14) and results in the development of diabetes (15). Both observations highlight a role for phosphatases, particularly PP-2B, in regulating insulin secretion. Furthermore, these studies show contrasting effects for transient *vs.* sustained inhibition of PP-2B on insulin secretion. This suggests that PP-2B activity must be tightly regulated to ensure that reversible phosphorylation events can occur repeatedly. This idea is supported by parallel studies in neurons where proteins involved in exocytosis/endocytosis are targets for reversible protein phosphorylation by PKA, PKC, and PP-2B. The dephosphorylation of these proteins by PP-2B allows for repeated exocytotic events stimulated by activation of the kinase (16).

In this study, we demonstrate that changes in insulin secretion are associated with phosphorylation of β cell substrates by PKA followed by dephosphorylation by PP-2B. These findings extend our previous observations that subcellular targeting of PKA through an A-Kinase Anchoring Protein (AKAP) facilitates hormone mediated insulin secretion (2). We now show that the coordinate action of PKA and PP-2B may be due in part to their recruitment into a signaling complex resulting in the activation of PP-2B by PKA, thus directly linking the activities of these two enzymes. These observations are consistent with our previous findings that demonstrate a role for the subcellular targeting of PKA through associations with AKAPs in GLP-1-mediated insulin secretion (2). We go on to show that the anchoring proteins, AKAP79/150 (17, 18) may coordinate the action of PKA and PP-2B by maintaining a signaling complex that regulates reversible phosphorylation events. Subcellular targeting of protein kinases and phosphatases has emerged as a prominent mechanism for regulating reversible phosphorylation events in neurons, T-Lymphocytes and cardiac myocytes (19–21). In this paper, we provide evidence that subcellular targeting of PKA and PP-2B regulates insulin secretion through reversible phosphorylation events in β cells.

Received May 4, 2000.

Address all correspondence and requests for reprints to: Dr. Linda Lester, Division of Endocrinology L-607, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97201. E-mail: lesterl@ohsu.edu.

* This work was supported by NIH Research Grant DK-02353 and American Diabetes Association Grant RA-0050 (to L.L.) and NIH Grant DK-5441 (to J.D.S.).

## Materials and Methods

### *Materials*

FK506 was a gift from Dr. John Rabkin, Liver, and Pancreas Transplantation, OHSU. Antibodies were obtained for synapsin 1 (Calbiochem,

La Jolla, CA), pan-calcineurin A (Veritas, Rockville, MA), anti-AKAP150 (Dr. J. D. Scott) and antiphosphoserine and antiphosphothreonine (Zymed Laboratories, Inc. San Francisco, CA). Insulin RIA kits were purchased from Linco Research, Inc. (St. Charles, MO). All other reagents including cyclosporin A, forskolin and rapamycin were obtained from Sigma (St. Louis, MO).

### *Preparation of primary islets and transfected RINm5F cells*

Pancreatic islets were isolated by collagenase digestion, hand picked, and plated in Falcon tissue culture dishes as previously described (22). Islets were maintained in culture for up to 5 days in RPMI 1640 containing 5 mM glucose and supplemented with streptomycin (100 $\mu$g/ml) and penicillin (100 IU/ml). RINm5F cells at passage 20–25 were transfected with the mammalian expression vectors pcDNA3, pcAKAP79, or pcHt31 using Lipofectamine (Life Technologies, Inc., Gaithersburg, MD). Transfected cell lines were selected by growth in media supplemented with G418. Cells were maintained in low glucose DMEM (1000 mg/liter D-glucose), with 10% FCS and 0.8 mg/ml G418. Expression of AKAP79 and Ht31 was monitored by immunoblotting.

### *Insulin secretion assay*

Insulin secretion from rat islets and RINm5F cells was measured in static culture using Krebs Ringer HEPES Buffer, KRBH (10 mM HEPES, pH 7.4, 0.1% BSA, 130 mM NaCl, 5.2 mM KCl, 1.3 mM $KH_2PO_4$, 1.6 mM MgCl, 2.8 mM $CaCl_2$, 20 mM $NaHCO_3$ and 2.8 mM glucose). Islets or RINm5F cells were pretreated for 30 min at 37 C with FK506, cyclosporin, rapamycin or DMSO as indicated. Cells were stimulated with 16.8 mM glucose, ± forskolin and ± FK506. Media was collected and centrifuged at 15,000 RPM × 10 min. The supernatant was stored at −20 C before determining insulin content by RIA using rat insulin as a standard (Linco Research, Inc.). Total insulin content was determined by incubating RINm5F cells and RINm5F cells expressing AKAP79 with 0.22M HCL and 95% ETOH at −20 C for 60 min. The protein content of the supernatant was determined using a colorimetric assay kit (Bio-Rad Laboratories, Inc.).

### *PP-2B and PKA assays*

RINm5F and rat islets were incubated with FK506 or DMSO for 30 min at 37 C in KRBH. The cells were stimulated with 16.8 mM glucose ± 10 $\mu$M forskolin for 30 min at 37 C. Following the stimulation, the cells were rinsed with PBS and incubated with 200 $\mu$l of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 2 $\mu$g/ml Pepstatin/leupeptin, 1 mM 4-(2-Aminoethyl)benzenesulfonyl Fluoride (AEBSF) and 10 $\mu$M IBMX) on ice for 60 min. Cells were scraped, pelleted, and sonicated. PKA activity was measured by the filter paper method of Corbin and Rieman (23) using Kemptide (LRRASLG) as a peptide substrate. PP-2B activity was measured in triplicate (24) in a 20 $\mu$l reaction mixture containing 40 mM Tris-HCL pH 7.5, 0.1 M KCl, 0.1 mM $CaCl_2$, 6 mM magnesium acetate, 0.5 mM DTT, 0.1 mg/ml BSA, 1.5 $\mu$M calmodulin and $^{32}$P-RII peptide (20 $\mu$M) as the substrate at 30 C.

### *Subcellular fractionation*

RINm5F cells were grown to near confluency ($5 \times 10^5$ cells per dish) and rinsed with KRBH. The cells were treated for 30 min with KRBH, 10 $\mu$M forskolin, or 10 $\mu$M forskolin + 10 $\mu$M FK506. The media was removed and the cells washed 3 times with PBS. The cells were scraped and lysed with lysis buffer [20 mM HEPES, pH 7.4, 0.2% Triton X-100, 20 mM NaCl, 5 mM EDTA, 1 mM DTT, 2 $\mu$g/ml leupeptin, 2 $\mu$g/ml of pepstatin, 1 mM benzamidine and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] and dounce homogenized. A supernatant and particulate fraction were obtained by centrifugation at 40,000 × *g* for 30 min. Protein concentration performed by a colorimetric assay (Bio-Rad Laboratories, Inc.). Twenty micrograms of protein from each fraction were separated by SDS-PAGE.

### *Affinity purification*

Cell lysates were used for immunoprecipitations, calmodulin purification, and cAMP purification. The cell lysates used for these experiments were prepared by plating RINm5F cells in 150-cm plates and culturing until approximately 80% confluency. The cells were washed twice with KRBH and incubated in either KRBH + DMSO or 10 $\mu$M FK506 for 30 min at 37 C. The cells were stimulated for the stated period of time. Cells were washed 3 times in PBS and incubated in lysis buffer (20 mM Tris-HCl, pH 7.9, 250 mM NaCl, 50 mM NaF, 5 mM EGTA, 0.1 mM PMSF, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml pepstatin, 1 mM Benzamidine and 1% vol/vol NP-40) for 15 min on ice. The lysate was cleared by centrifugation at 15,000 rpm for 10 min. Lysates were incubated with either 2 $\mu$g of antibody (for the immunoprecipitations), calmodulin Sepharose (for the calmodulin purification) or cAMP agarose (for the cAMP affinity purification) as previously described (2). Affinity columns were washed 7 times and eluted by boiling with SDS-PAGE buffer (unless stated otherwise). The C-subunit was eluted from the protein A Sepharose column by incubating the column with pH 7.4, 20 mM HEPES buffer containing 75 mM cAMP for 15 min at room temperature. PKA assays were performed as described above. All other protein elutions were subjected to SDS-PAGE electrophoresis, transferred to PVDF membranes and probed for the specified protein.

### *Phosphoprotein determination*

RINm5F cells were grown to near confluency in 150-mm dishes. The cells were rinsed in prewarmed KRBH without bovine-serum albumin and treated with DMSO or FK506 for 30 min at 37 C. The media was replaced with KRBH containing 10 $\mu$M IBMX and 16.8 mM glucose plus 10 $\mu$M forskolin or 100 $\mu$M FK506 if indicated for 30 min. At the end of the incubation period, cells were rinsed with PBS, scraped and pelleted by centrifugation. The cells were lysed by adding 100 $\mu$l of RIPA buffer containing 2 $\mu$g/ml pepstatin/leupeptin, 1 mM AEBSF and 10 $\mu$M IBMX on ice for 60 min followed by 15 sec sonication (×3). A 30 $\mu$l aliquot of cell lysate was denatured in Laemmli sample buffer and subjected to SDS-PAGE. Proteins were transferred to PVDF membranes and blocked for a minimum of 1 h in Tris-buffered saline containing 50 mM NaCl, 0.1% (vol/vol) Tween 20 and 5% (wt/vol) BSA. Blots were incubated with primary antibody at the recommended concentration of 1 $\mu$g/ml (Zymed Laboratories, Inc., San Francisco, CA). Blots were incubated with secondary antibody for 2 h at room temperature. Proteins were visualized by chemiluminescence. *In vitro* phosphorylation of synapsin 1 immunoprecipitates was performed in a 30 $\mu$l reaction volume containing 5 $\mu$Ci $^{32}$P $\gamma$-ATP and 0.07 $\mu$g PKA catalytic subunit for 60 min at 30 C in the presence or absence of 15 $\mu$M PKI. The reaction was stopped by adding 5 $\mu$l of 0.5 M EDTA and boiling in SDS-PAGE sample buffer. Phosphoproteins were identified by autoradiography.

### *Confocal microscopy*

RINm5F cells were grown on no. 1 coverslips until 30% confluent. The cells were rinsed in PBS three times and fixed with 3.7% formaldehyde for 5 min at 20 C. After washing, the cells were permeablized with ice-cold acetone for 1 min. The cells were washed and blocked with PBS containing 0.1% BSA for 10 min. AKAP 79 was visualized using a rabbit polyclonal antibody (J. D. Scott) and a Texas-Red conjugated antirabbit secondary antibody. The regulatory subunit of PKA was visualized using a goat-anti-RII antibody (J. D. Scott) and a FITC conjugated antigoat secondary antibody. The cells were incubated with the primary and secondary antibodies for 60 min at 20 C. The cover-slips were mounted with Prolong (Molecular Probes, Inc.) and confocal sections were taken using a laser-scanning confocal microscope (Carl Zeiss).

### *Statistical analysis*

Data are represented as means ± SE. The data were evaluated for significance by a two-sided nonpaired Student's *t* test using Prism software (GraphPad Software, Inc., San Diego, CA).

## Results

### *Inhibition of PP-2B increases cAMP mediated insulin secretion*

The immunosuppressant drugs FK506 and cyclosporin A (CsA) are selective inhibitors of PP-2B activity and are often

used to investigate the cellular function of PP-2B (25). Using these drugs we determined the effects of PP-2B signaling in primary intact rat islets and transformed $\beta$ cells (RINm5F). Rat islets were preincubated with FK506 for 30 min at 37 C and static insulin secretion was measured by RIA following 30 min of stimulation with either glucose or glucose + forskolin. FK506 treatment increased forskolin stimulated insulin secretion by 2.5-fold $\pm$ 0.5 (n = 6) over controls (Fig. 1A). Likewise, transformed $\beta$ cells, RINm5F cells, treated with FK506 and forskolin had a 2.11-fold $\pm$ 0.3 (n = 6) increase in insulin secretion over RINm5F cells treated with forskolin (Fig. 1B). Insulin secretion was increased to a similar magnitude [(2.48-fold $\pm$ 0.7 (n = 3)] using a more physiologic agonist, GLP-1 (1 nM) (Fig. 1C). Importantly, insulin secretion from rat islets was increased to a similar extent [2.6-fold $\pm$ 0.5 (n = 4)] upon application of another PP-2B inhibitor, CsA (Fig. 1D). Further control experiments were performed using a structurally related macrolide, rapamycin, that binds the immunophilin FKBP12 but does not inhibit PP-2B (26, 27). As expected, pretreatment of rat islets with excess rapamycin (10 $\mu$M) blocked the FK506 affect on cAMP-mediated insulin secretion but rapamycin did not affect glucose-mediated insulin secretion independent of FK506 (Fig. 1E). This observation is consistent with a role for rapamycin as a competitor with FK506 for binding to FKBP12 (28, 29). Collectively, these experiments confirm that short-term inhibition of PP-2B activity by FK506 or CsA is responsible for an increase in cAMP-mediated insulin secretion from rat islets or transformed $\beta$ cells.

Although transient inhibition of PP-2B by CsA increases glucose-mediated insulin secretion (13), prolonged inhibition of PP-2B inhibits insulin release (14, 30). Therefore, rat islets were pretreated with FK506 over a time course of 24 h to determine whether cAMP-mediated insulin secretion was dependent on the duration of PP-2B inhibition (Fig. 2). There was no significant difference in basal secretion between the islet groups (Fig. 2, zero time point). However, treatment of islets with FK506 for 1 h increased forskolin and glucose mediated insulin secretion [2.7-fold $\pm$ 0.4 (n = 4)] over the non-FK506-treated islets (Fig. 2 and 1 h time point). However, three hours of FK506 treatment inhibited cAMP mediated insulin release and this inhibition was maintained during the remaining 24 h of FK506 treatment (Fig. 2). Therefore, we confirmed that prolonged inhibition of PP-2B with FK506 results in a loss of insulin secretion (14, 15). However, we now demonstrate that the FK506-mediated inhibition of in-

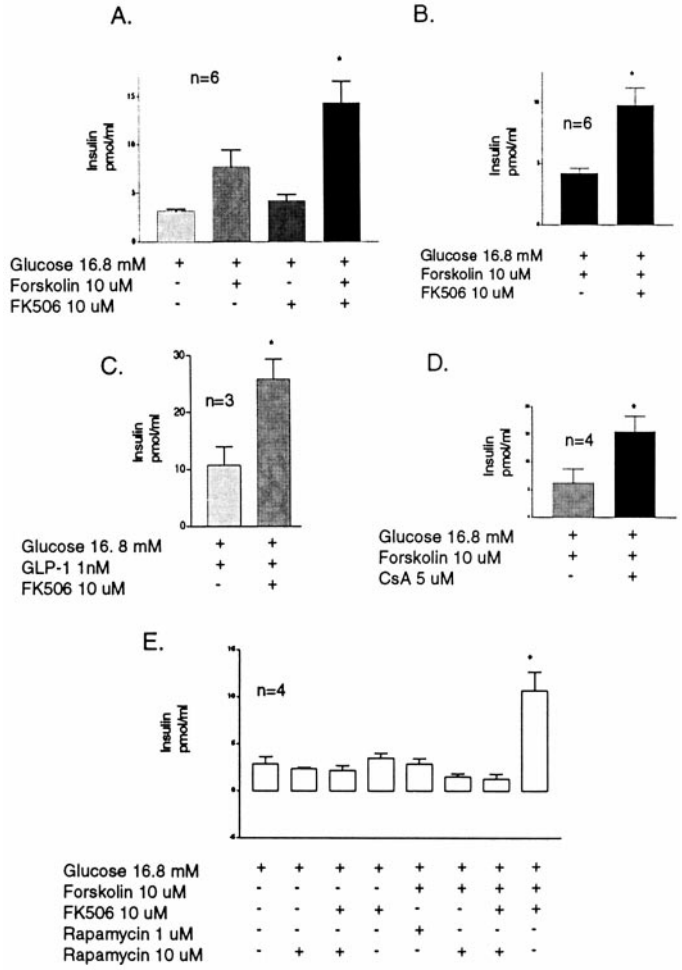


FIG. 1. Inhibition of PP-2B increases cAMP mediated insulin secretion. A, Native rat islets (20 islets per well) were treated with 10 $\mu$M FK506 for 30 min followed by 30 min of stimulation with 16.8 mM glucose $\pm$10 $\mu$M forskolin. B, RINm5F cells ($1 \times 10^5$ per well) were treated with 10 $\mu$M FK506 for 30 min followed by 30 min of treatment with 10 $\mu$M forskolin. C, Insulin secretion from static cultures of rat islets treated with 1 nM GLP-1 and 10 $\mu$M FK506 for 30 min. D, RINm5F cells treated with 5 $\mu$M CsA for 30 min followed by 30 min of treatment with 10 $\mu$M forskolin. E, Native rat islets (20 per well) were treated with FK506, rapamycin, or KRBH for 30 min followed by stimulation with 16.8 mM glucose $\pm$ 10 $\mu$M forskolin for 30 min. All treatment groups were performed in triplicate, the number of experiments is indicated for each *bar graph*. Cell treatments are indicated below each *bar*. Insulin was measured by RIA in the culture media and data are shown as pmol/ml. Statistical significance was determined by *t* test usingPrism, GraphPad Software, Inc. *, $P < 0.01$ for immunosuppressant and forskolin treatment *vs.* forskolin alone.

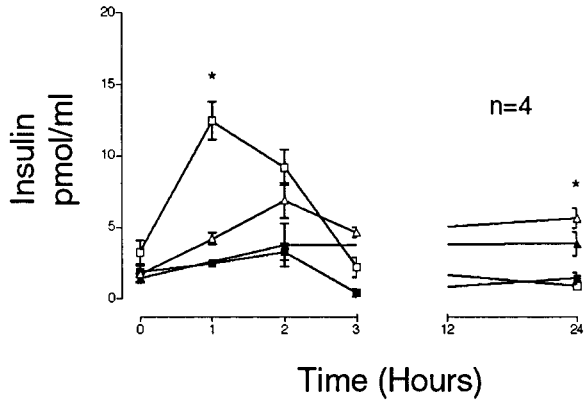


FIG. 2. Prolonged treatment with FK506 inhibits forskolin stimulated insulin secretion. Native rat islets (20 per well) were pretreated with 10 $\mu$M FK506 or DMSO for 30 min. Media was collected before stimulation with glucose or forskolin and is shown as the zero time point. Other islets were pretreated with 10 $\mu$M FK506 for 1, 2, 3, and 24 h. Islets were stimulated with 16.8 mM glucose $\pm$ 10 $\mu$M forskolin for 30 min. Mean $\pm$ SE insulin secretion for glucose only (▲), forskolin only (△), Glucose + FK506 (■) and Forskolin + FK506 (□) are shown. Statistical significance was determined by *t* test using Prism, GraphPad Software, Inc. *, $P < 0.01$ for forskolin and FK506 *vs.* forskolin alone.

sulin secretion occurs with nonnutrient stimuli (cAMP) as well as with nutrient stimuli (glucose).

*Synapsin 1 is a target for PKA/PP-2B reversible phosphorylation*

Although the transient inhibition of PP-2B enhanced forskolin stimulated insulin secretion the molecular mechanism of this process is unknown. We hypothesized that certain $\beta$ cell proteins would be phosphorylated in response to forskolin activation of PKA and dephosphorylated by PP-2B. Inhibiting PP-2B would temporarily increase the phosphorylation state of these proteins resulting in increased insulin secretion. To evaluate this possibility, we identified proteins reversibly phosphorylated by PKA and PP-2B using antiphosphoserine and antiphosphothreonine antibodies as previously described (31). Forskolin stimulation of RINm5F cells decreased the serine phosphorylation of an 84-kDa protein whereas forskolin and FK506 treatment increased the serine phosphorylation (Fig. 3, A and B). No change in phosphorylation was identified in phosphothreonine containing proteins following treatment with either forskolin or FK506 (data not shown). Therefore, the 84-kDa phosphoprotein fulfilled the criterion for a $\beta$ cell PKA and PP-2B substrate. The mobility of this protein was similar to synapsin 1, a recently identified 84-kDa $\beta$ cell phosphoprotein (32) that is a substrate for PKA (33) and PP-2B (13).

To confirm that our 84-kDa phosphoprotein was synapsin 1, we immunoprecipitated synapsin 1 from RINm5F lysates under a variety of treatment conditions. Western blot analysis of each immunoprecipitate confirmed that equal amounts of synapsin 1 protein were present in each lane (Fig. 3, *lower panel*). Serine phosphorylation of the immunoprecipitated band was decreased following forskolin treatment, whereas forskolin and FK506 treatment increased the serine phosphorylation (Fig. 3C, lanes 1 and 3). Differences in the serine phosphorylation of the synapsin 1 protein were determined by densitometry (Fig. 3D), and these changes paralleled those found in whole RINm5F lysates (Fig. 3A). Collectively, these results suggest that synapsin 1 underwent reversible phosphorylation following forskolin treatment of $\beta$ cells.

To confirm that phosphorylation of synapsin 1 was mediated through the activation of PKA, experiments were repeated in the presence of the PKA inhibitor, PKI (34). Synapsin 1 immunoprecipitates were incubated with the catalytic subunit of PKA in the presence or absence of FK506 and

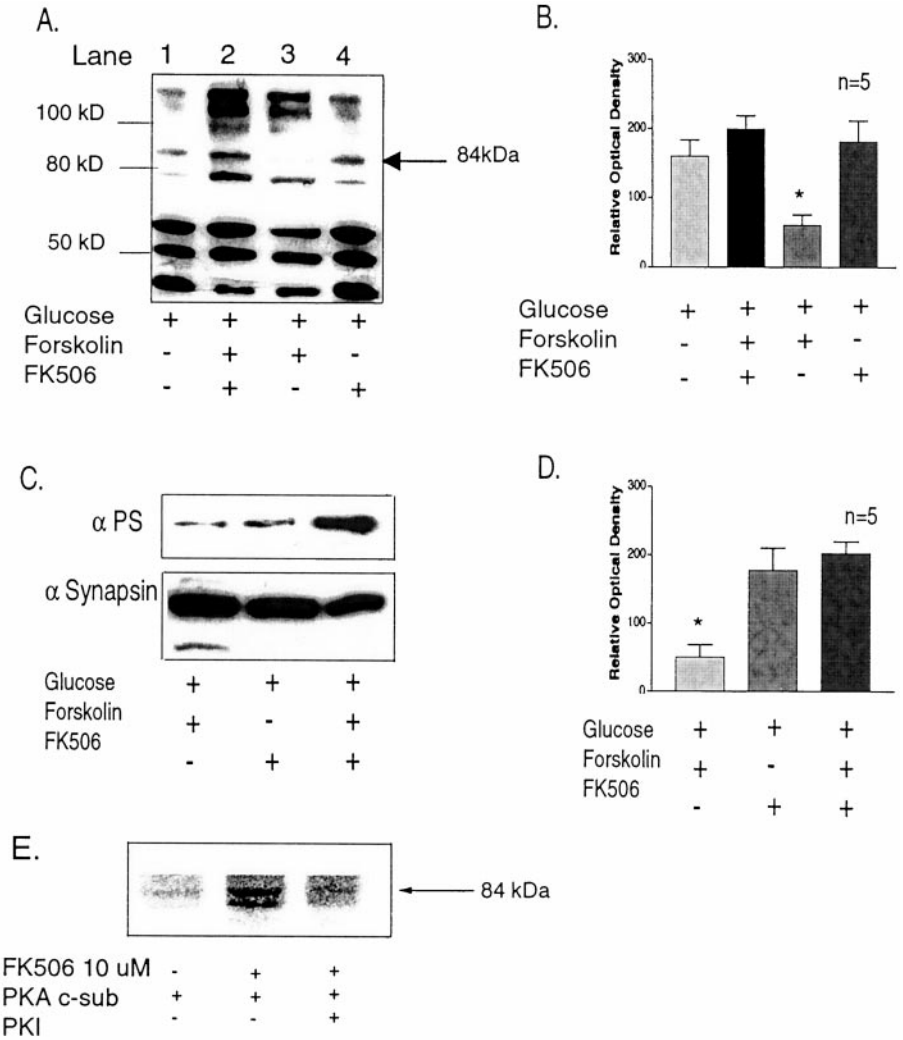


FIG. 3. Synapsin 1 is a target for PKA/PP-2B reversible phosphorylation. A, Antiphosphoserine blot of RINm5F lysates treated with forskolin or FK506. MW markers are indicated on the left. An *arrow* indicates an 84-kDa band that changes with treatment. B, Changes in the 84-kDa band intensity were quantitated by scanning densitometry using NIH image software. *, $P < 0.01$ for forskolin and FK506 *vs.* forskolin alone. C, Synapsin 1 immunoprecipitates were immunoblotted with the antiphosphoserine antibody in the *upper panel* ($\alpha$PS) or antisynapsin 1 antibody in the *lower panel* ($\alpha$Synapsin). D, Changes in the phosphorylation of synapsin 1 were quantitated by densitometric analyses of 5 individual immunoblots using NIH image. *, $P < 0.01$ for forskolin and FK506 *vs.* forskolin alone. E, Radiolabeled *In vitro* phosphorylation of synapsin 1 immunoprecipitated from RINm5F cell lysates in the presence or absence of PKI. An *arrow* indicates an 84-kDa band corresponding to synapsin 1. Cell treatments are indicated below the blot or graph. *, $P < 0.01$ for forskolin and FK506 *vs.* forskolin alone.

PKI. Synapsin 1 phosphorylation was greater in the presence of FK506 and PKA than when PKI was also present (Fig. 3E). These findings suggest that activation of PKA is necessary for the forskolin-mediated phosphorylation and dephosphorylation of β cell synapsin 1.

*Activation of PKA increases PP-2B activity*

Given the observation that the activities of PKA and PP-2B were coordinated resulting in the reversible phosphorylation of synapsin 1, we hypothesized that activation of PKA may result in increased PP-2B activity. To understand the interaction between PP-2B and PKA activities in β cells we measured kinase and phosphatase activities in FK506 and forskolin treated RINm5F whole cell lysates. FK506 treatment decreased both basal and forskolin treated cellular PP-2B activity (Fig. 4A). However, application of forskolin and activation of PKA was associated with a 2-fold ($P < 0.001$) increase in cellular PP-2B activity compared with basal levels (Fig. 4A). As expected, forskolin treatment also increased PKA activity (Fig. 4B). However, inhibition of PP-2B activity did not significantly change PKA activity (Fig. 4B). These data support a role for PKA activation in the activation of PP-2B; however, the cellular mechanism that coordinates the activities of PKA and PP-2B is currently unknown. Previous studies have suggested that colocalization of PKA and PP-2B can regulate their enzymatic activities (35).

To evaluate a role for enzyme targeting in coordinating the activities of PKA and PP-2B, we treated RINm5F cells with cell soluble PKI or the peptide Ht31 that disrupts PKA anchoring (36). Both inhibiting PKA targeting (Fig. 4C, lane 3) and inhibiting the catalytic activity of PKA (Fig. 4C; lane 4) blocked the dephosphorylation of the 84-kDa band seen following forskolin application (Fig. 4C, lane 2). These data suggest that targeted PKA is needed for the activation of PP-2B. To confirm this finding, we measured forskolin stimulated PP-2B activity in RINm5F cells constitutively expressing the anchoring inhibitor protein, Ht31 (Fig. 4D). Forskolin stimulated PP-2B activity (Fig. 4D) was significantly lower in the Ht31 cells compared with RINm5F cells transfected with vector alone (pcDNA). Together, these data support a role for targeted PKA in PP-2B activation and reversible phosphorylation of synapsin 1 in β cells.

*Association of PP-2B and PKA in pancreatic β cells*

In neurons, both PKA and PP-2B are colocalized by the PKA targeting protein AKAP79/150 (18, 20). Given the as-

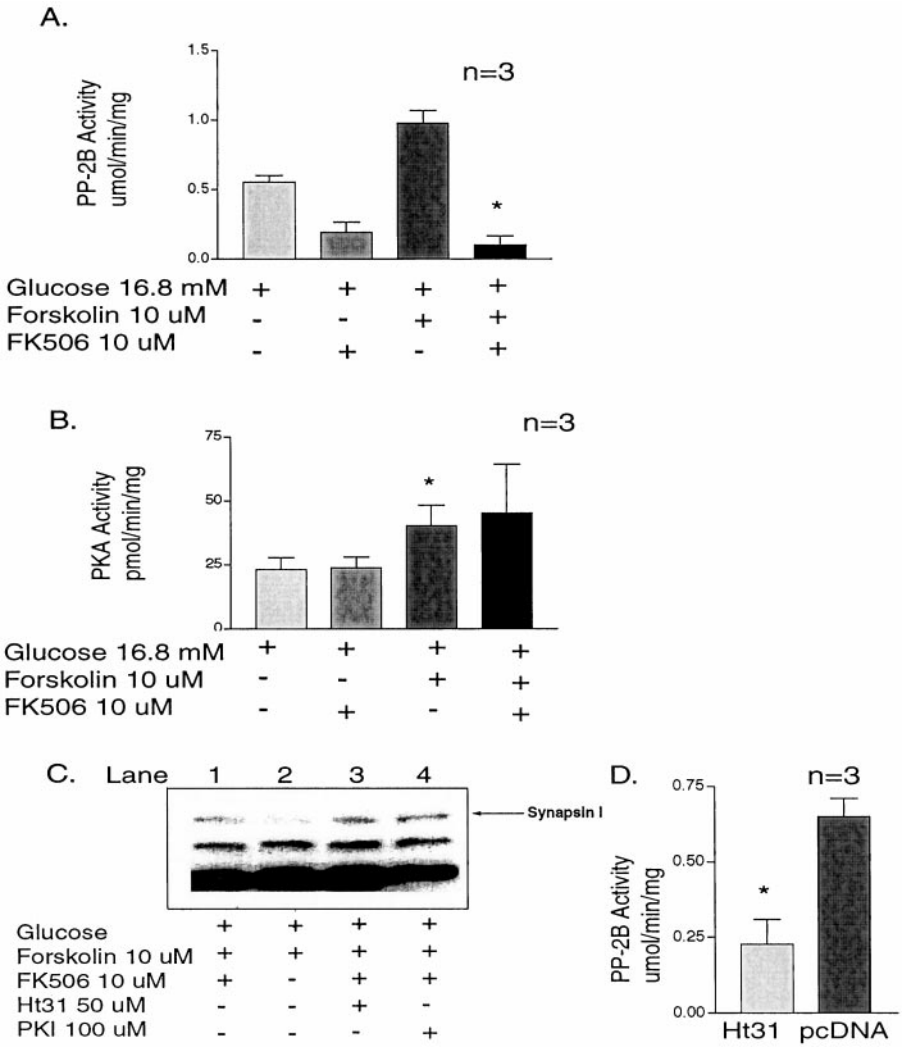


FIG. 4. Forskolin treatment increases PP-2B activity. A, PP-2B activity was assayed in triplicate as described by Blumenthal *et al.* (27) using $^{32}$P-RII peptide as the substrate in RINm5F cell lysates following 30 min of forskolin stimulation. Mean ± SE from three separate experiments is shown in μmol/min/mg. *, $P < 0.005$ for forskolin and FK506 *vs.* forskolin alone. B, PKA catalytic activity was assayed in three separate experiments using Kemptide as the substrate. Cell treatments are indicated below each *bar*. *, $P < 0.01$ for forskolin and FK506 *vs.* FK506 alone. C, Antiphosphoserine blot of RINm5F cell lysates treated with forskolin, FK506, the PKA anchoring inhibitor protein Ht31 or the PKA catalytic inhibitor, PKI. D, Forskolin stimulated PP-2B activity measured in lysates of RINm5F cells overexpressing the PKA anchoring inhibitor protein, Ht31. *, $P < 0.01$ for Ht31 OE *vs.* pcDNA transfected cells. Differences between the means were determined by nonpaired T testing, GraphPad Software, Inc. (Prism).

sociation we found between PKA and PP-2B in β cells, we hypothesized that AKAP79/150 may target these two enzymes in pancreatic β cells. To investigate this possibility, we looked for an association between these two enzymes. Initially, we identified AKAP150, the rat homologue of AKAP79, in pancreatic β cells by calmodulin affinity chromatography. AKAP150 was identified in the eluate by both a radiolabeled RII binding assay (RII overlay) and by immunoblotting (Fig. 5A). AKAP150 is the upper band seen in both the immunoblots and radiolabeled overlays whereas the lower band is a presumed breakdown product. Subsequently, we identified an association between PP-2B and PKA in β cells using two complementary biochemical methods. First, we isolated PKA binding proteins from RINm5F lysate using a cAMP affinity purification method as previously described (36). PP-2B was identified in the lysate, flow-through (FT) and eluate by immunoblotting with a specific PP-2B antibody (Fig. 5B). Secondly, we immunoprecipitated PP-2B from RINm5F lysates and measured PKA activity. PKA specific activity was approximately 30-fold ± 2 (n = 4) greater in the PP-2B immunoprecipitate than in a preimmune immunoprecipitate (Fig. 5C). Finally, AKAP150 was identified in the eluate of a PP-2B immunoprecipitation from RINm5F cells but not in the preimmune elution (Fig. 5D). Together, these data strongly support an association between a subset of PKA and PP-2B in β cells probably via AKAP79/150.

### *AKAP79 inhibits PP-2B in pancreatic β cells*

If AKAP targeting of PKA and PP-2B was involved in the regulation of insulin secretion, then overexpression of the AKAP would alter the effects of PKA and PP-2B on β cell function. To test this hypothesis, we expressed AKAP79, the human homologue of AKAP150, in RINm5F cells. RINm5F cells expressing AKAP79 (79OE) or vector alone (pcDNA)

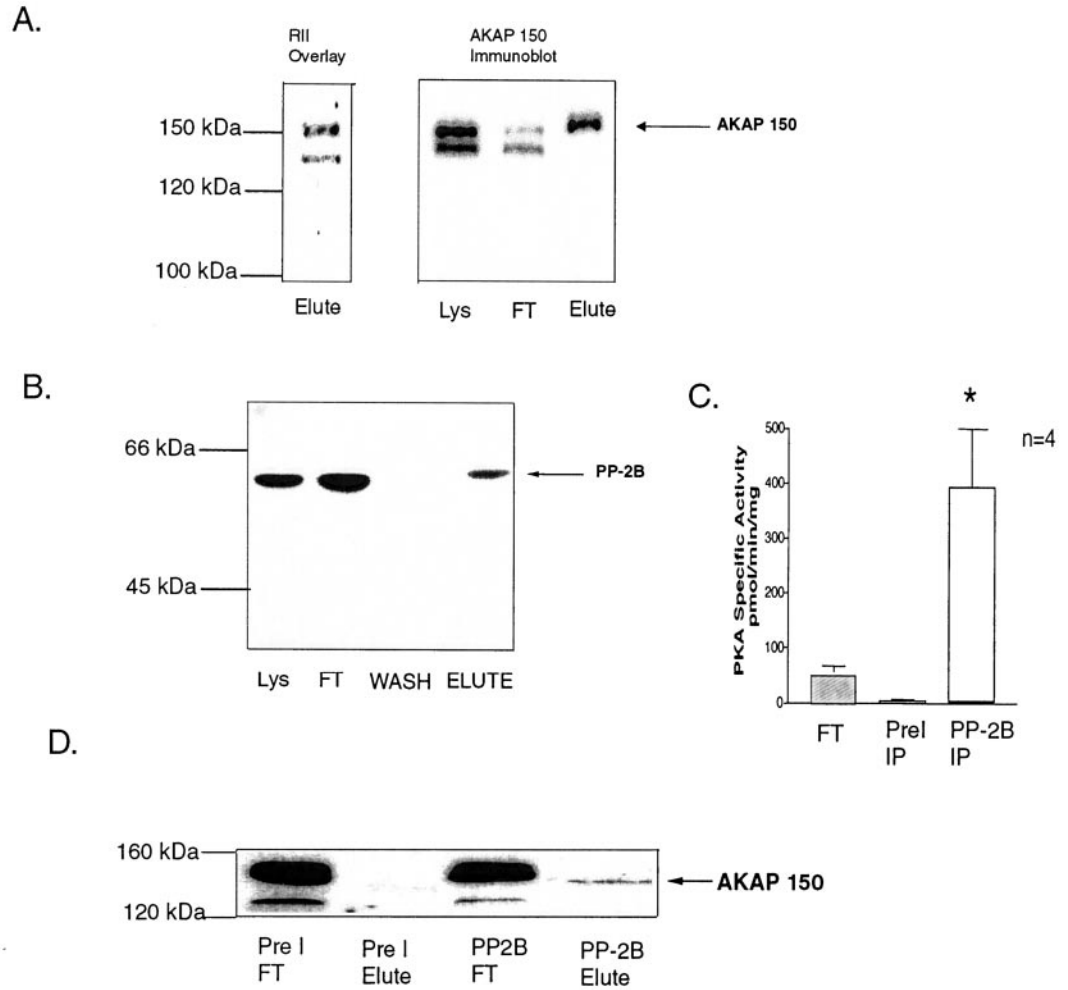


FIG. 5. Association of PKA and PP-2B in pancreatic β cells. A, Purification of calmodulin binding proteins from RINm5F cells. RIN cell lysates (Lys), and calmodulin column flow-through (FT) and eluates were subjected to SDS-PAGE. Blots were probed with either radiolabeled RII α (RII Overlay) or anti-AKAP150 antibody (AKAP 150 Immunoblot). *Arrow* indicates AKAP150. MW markers are indicated along left side of blot. B, cAMP binding proteins were purified from RINm5F cells by cAMP affinity chromatography. PP-2B was detected in the lysate (Lys), flow through (FT) and in the cAMP elution (Elute). An *arrow* indicates the A subunit of PP-2B. C, PP-2B was immunoprecipitated from RINm5F lysates with affinity-purified antibodies to PP-2B A subunit. PKA specific activity, mean ± SE, was measured in the cAMP eluate using Kemptide as the substrate. *, $P < 0.05$ for Pre-I IP *vs.* PP-2B IP. D, PP-2B was immunoprecipitated from RINm5F cell lysates using anti-PP-2B polyclonal antibody or preimmune sera (Pre I) as described above. AKAP150 was identified by immunoblotting in the Pre-I FT, PP-2B FT, and PP-2B elute.

were treated with forskolin and FK506 as described in Fig. 1. The RINm5F cells expressing AKAP79 had lower forskolin and forskolin + FK506 (Fig. 6A) stimulated insulin secretion than did wild-type RINm5F or pcDNA controls. Because the 79OE cells had lower forskolin mediated insulin secretion, we calculated the fold increase in insulin secretion of FK506 + forskolin over forskolin alone (Fig. 6B). 79OE cells had significantly lower FK506 fold insulin secretion than did either RIN wild-type (wt) or vector controls (pcDNA) ($P < 0.05$) suggesting that at least part of the 79OE phenotype was related to changes in PP-2B activity. Other investigators have shown that AKAP79 binds to PP-2B via residues 108–427 resulting in an inhibition of PP-2B activity (20, 35). Because constitutive inhibition of PP-2B by FK506 treatment can also decrease forskolin stimulated insulin secretion (as shown in Fig. 2) we hypothesized that constitutive expression of AKAP79 would inhibit PP-2B activity. To test this hypothesis, we measured PP-2B activity in the lysates of cells constitutively expressing AKAP79. Expression of AKAP79 decreased PP-2B activity compared with RINm5F or pcDNA lysates (Fig. 6C). This effect was not secondary to differences in the amount of PP-2B found in the cell lysates as determined by immunoblotting with specific PP-2B antibody (Fig. 6D). Changes in insulin messenger RNA and insulin content have also been associated with immunosuppressant treatment of islets or $\beta$ cells (30, 37). Therefore, to determine if the diminished insulin secretion observed in the 79OE cells was associated with changes in insulin content, we measured total insulin content in the wild-type RINm5F, pcDNA transfected RIN, and 79 transfected RIN cells. We found no statistical difference in insulin content of 79OE cells compared with either the RIN wild-type or RIN pcDNA cells (Fig. 6E). Therefore, the decrease in forskolin and FK506 mediated insulin secretion in AKAP79 expressing cells was not secondary to significant decreases in insulin content. Of interest, the RIN wild-type cells have slightly diminished insulin secretion, lower PP-2B activity and insulin content compared with the pcDNA transfected cells. The reason for this difference is not known, however, we suspect that differences in the tissue culture conditions (*i.e.* the presence or absence of the selecting agent, G418) or passage number could have produced these changes (38). Importantly, the 79OE and pcDNA transfected cells were treated identically therefore, the pcDNA cell line is the appropriate control for the 79OE cells. Despite the differences in cell culturing, the 79OE cells had diminished FK506 and forskolin-mediated insulin secretion and PP-2B activity compared with both the RIN Wt and pcDNA cells. Taken together this data supports a role for AKAP150/79 as an endogenous inhibitor of PP-2B activity and suggests that targeting of PP-2B may regulate its activation.

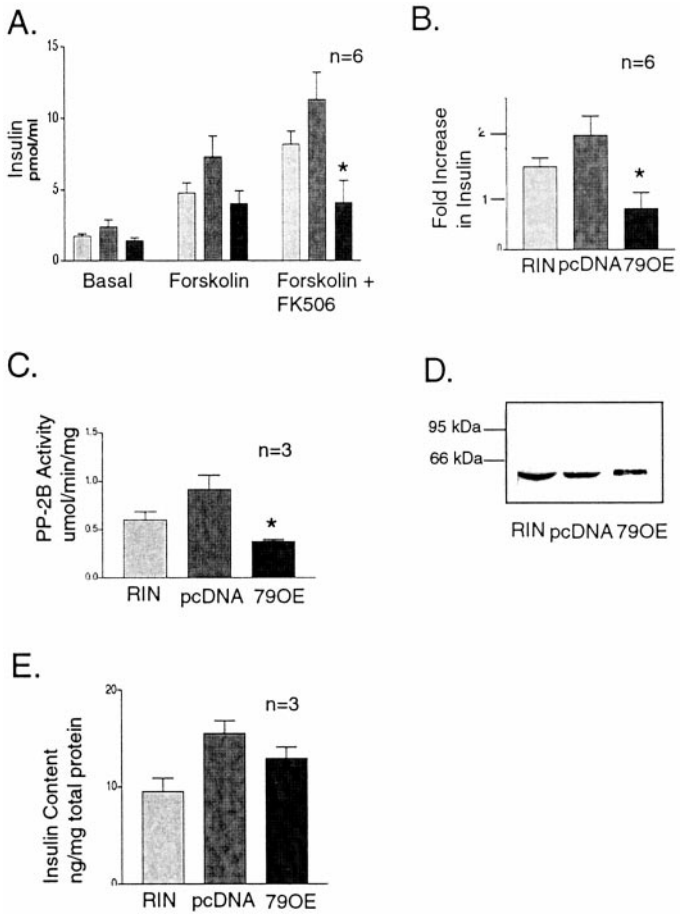


FIG. 6. Overexpression of AKAP79 inhibits PP-2B and blocks FK506 effects in $\beta$ cells. RINm5F cells overexpressing AKAP 79 were treated with forskolin or forskolin + FK506. A, Thirty minute static insulin secretion in response to 10 $\mu$M forskolin or forskolin + FK506. Cell treatments are indicated below each *bar*. Mean ± SE for RINm5F wild-type cells (□), RIN transfected with pcDNA vector (■) and RIN transfected with AKAP79 (■) are shown. B, Fold increase in FK506-mediated insulin secretion over forskolin alone. Cell type is indicated below *bar*. C, Basal (nonstimulated) PP-2B activity was measured in cells expressing RIN wild-type (RIN), vector alone (pcDNA) or AKAP79 (79OE) in triplicate as described earlier. D, Cell lysates were separated by SDS-PAGE and transferred to PVDF. PP-2B was identified in the lysates by immunoblotting using an anti-panA calcineurin (PP-2B) antibody. Molecular weight markers are shown on the *left*. E, Total insulin content in acid/alcohol extraction of RIN, pcDNA and 79OE cells was measured by RIA. Insulin content was expressed per mg of total cellular protein. The number of experiments is indicated for each bar graph. Statistical significance was determined by *t* test using Prism, GraphPad Software, Inc. *, $P < 0.01$ for 79OE cells *vs.* the pcDNA cells.

### *Subcellular targeting by AKAP79*

We have previously shown that the subcellular location of PKA can alter insulin secretion in response to GLP-1 (39) and hypothesized that AKAP79 expression could produce similar effects on insulin secretion through changes in PKA targeting. To test this, we determined the subcellular location of PKA and AKAP79 in RINm5F cells expressing AKAP79 by confocal fluorescent microscopy compared with wild-type RINm5F cells. The regulatory subunit of PKA is predominantly seen in a perinuclear distribution in wild-type RINm5F cells (Fig. 7A). In contrast, the regulatory subunit of PKA is found at the cell periphery in the 79OE cells. The altered subcellular localization of PKA is associated with increased AKAP79 targeting at the cell periphery. Minimal signal for AKAP79 is seen in the wild-type RIN cells consistent with the low levels of expression of AKAP150 in these cells and the diminished affinity of the AKAP79 antibody for AKAP150. Together, these data show that AKAP79 expres-

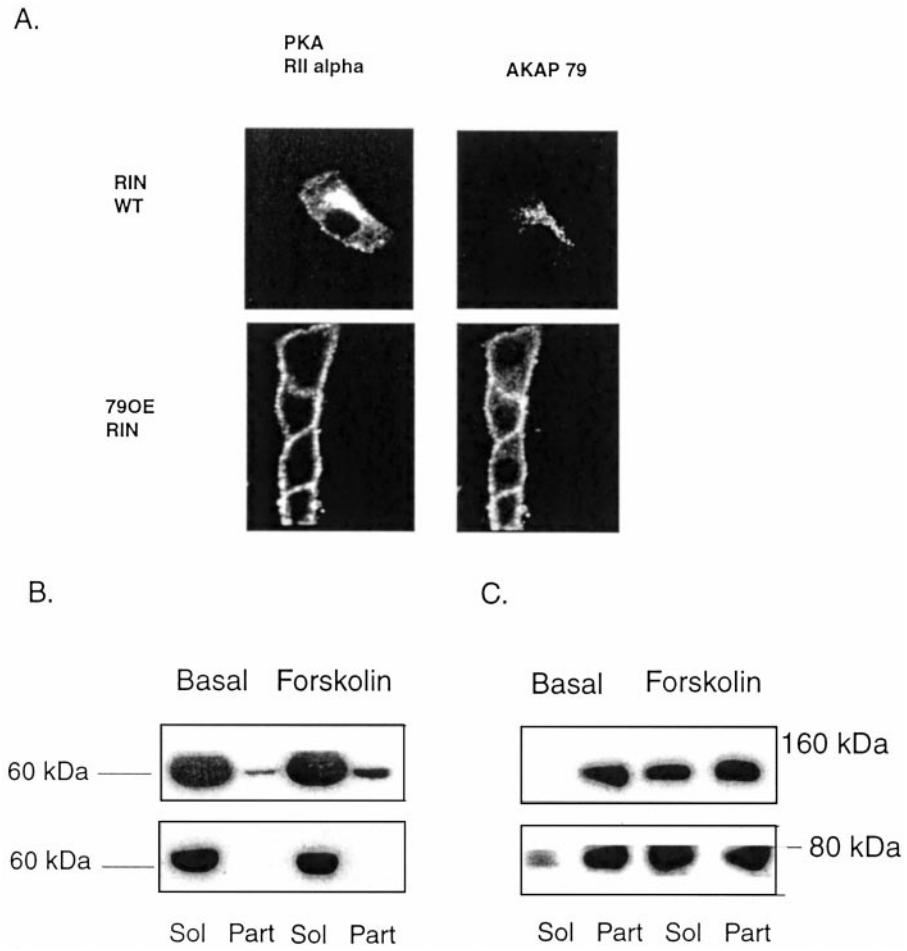


FIG. 7. Subcellular targeting by AKAP79. A, AKAP79 and the regulatory subunit (RII) of PKA are identified in RINm5F cells transfected with AKAP79 (79 OE RIN) or in wild-type, nontransfected RIN (RIN WT) using fluorescent tagged secondary antibodies to AKAP79 (Texas Red) and RII (FITC). B, Cytosolic and particulate fractions of RINm5F cells (*upper panel*) or RINm5F cells expressing AKAP79 (*lower panel*) treated with KRBH or forskolin were prepared by centrifugation and separated by SDS-PAGE. PP-2B was identified by immunoblotting using a polyclonal anticalcineurin A antibody. C, Cytosolic and particulate fractions were prepared as above from RINm5F cells (*upper panel*) or RIN79OE cells (*lower panel*) AKAP150 (*upper panel*) or AKAP79 (*lower panel*) were identified by immunoblotting using specific antibodies to AKAP150 and AKAP79, respectively.

sion alters the distribution of PKA. However, this does not completely explain the change in insulin secretion because expression of AKAP 18 also targets PKA to the periphery of $\beta$ cells but results in an increase in PKA mediated insulin secretion (39). Therefore, we believe that AKAP79 targeting may also affect the subcellular location of PP-2B, thus producing the decrease in insulin secretion. To evaluate this possibility, we measured AKAP150 and PP-2B in soluble and particulate fractions of both basal and forskolin stimulated RINm5F cells (Fig. 7, B and C, *upper panels*). PP-2B was found predominately in the soluble fraction. However, forskolin treatment resulted in a greater portion of PP-2B in the particulate fraction (Fig. 7B, *upper panel*). AKAP150 is predominately found in the particulate fraction with a portion moving into the soluble fraction following forskolin treatment (Fig. 7C, *upper panel*). Expression of AKAP79 appears to diminish the movement of PP-2B to the particulate fraction (Fig. 7B, *lower panel*), whereas AKAP79 was found in both the soluble and particulate fractions in the AKAP79 OE cells (Fig. 7C, *lower panel*). The increased amount of AKAP79 in the soluble fraction may be associated with the decrease in PP-2B activity and the decreased movement of PP-2B to the particulate fraction in the 79 OE cells. This suggests that AKAP targeting of PKA, depending upon cellular conditions, could modify the subcellular movement of PP-2B and alter the interaction between the two signaling enzymes.

## Discussion

Reversible phosphorylation serves as a mechanism to couple signals from various stimuli to insulin secretion in pancreatic $\beta$ cells (12). To understand the role of reversible phosphorylation in $\beta$ cells, it is important to identify both the enzymes involved and the mechanisms regulating the activities of the enzymes. In this paper, we show that PKA activation of PP-2B results in the reversible phosphorylation of $\beta$ cell proteins including synapsin 1 and regulates insulin release. Furthermore, our data supports a role for AKAP targeting of these enzymes in the regulation of reversible phosphorylation events involved in insulin secretion.

We initially observed a synergistic effect of forskolin and FK506 on insulin secretion suggesting that an increase in PKA and a decrease in PP-2B activities regulate insulin release. This finding is consistent with the previous observations that acute activation of kinases or inhibition of phosphatases increased insulin secretion (12, 40). In addition, we found that prolonged (>3 h) PP-2B inhibition results in suppressed PKA mediated insulin secretion reflecting a possible role for PP-2B in resensitizing the cell for repeated stimulation. Previous studies have demonstrated that dephosphorylation of $\beta$ cell proteins involved in insulin exocytosis results in desensitization of the cell to additional stimulations (41). Chronic inhibition of PP-2B could desensitize $\beta$ cells

resulting in decreased insulin secretion. This would explain the long-term effects of immunosuppressant drugs (CsA and FK506) on decreased insulin release and development of type 2 DM (15). Furthermore, we showed that the effect of FK506 on insulin secretion could be blocked by cotreatment with another immunosuppressant, rapamycin (Sirolimus). Although the effect of rapamycin in $\beta$ cells is not well known, both rapamycin and FK506 must bind to FKBP12 before they can activate their target proteins, mTOR and PP-2B, respectively (42). Therefore, rapamycin may compete with FK506 for binding to FKBP12 preventing inhibition of PP-2B by FK506 (29). The interaction between these immunosuppressant drugs on $\beta$ cell function is quite important because recent studies have shown that mixtures of immunosuppressants may affect $\beta$ cell function following islet cell transplantation (43). Understanding the targets for these drugs in $\beta$ cell will provide important insight into the use of immunosuppressants for future islet transplantation.

Our data clearly demonstrates that FK506 treatment inhibits PP-2B activity leading to the persistent phosphorylation of $\beta$ cell proteins including synapsin 1. Synapsin 1 has recently been identified as a $\beta$ cell protein (32) and is phosphorylated by PKA and dephosphorylated by PP-2B (13, 33, 44). Therefore, it is not surprising to find changes in the phosphorylation state of synapsin 1 resulting from PKA activation and PP-2B inhibition. However, because we did not perform peptide mapping of synapsin 1, we cannot distinguish between direct PKA phosphorylation of synapsin 1 *vs.* an indirect effect of PKA activation on other kinases, such as CaM K II. Our data does indicate that reversible phosphorylation of synapsin 1 was dependent upon PKA activation of PP-2B, suggesting that the activities of these enzymes were closely linked. Although the specific mechanism linking the activities of PKA and PP-2B is currently unknown, there are two models that could explain this. First, PKA activation could increase intracellular calcium and calmodulin concentrations and subsequently activate PP-2B. Although such a mechanism is plausible because PKA has documented effects on intracellular calcium levels (5), we controlled the concentration of calcium and calmodulin in our evaluation of PP-2B activity thereby minimizing the effect of PKA mediated increases in calcium. Secondly, PKA could directly phosphorylate PP-2B thus decreasing its phosphatase activity. PP-2B is a substrate for PKA phosphorylation, but this is readily blocked by calmodulin, which was present in our assay mixture (45). Therefore, neither model adequately explains the forskolin stimulated PP-2B activity we found in our $\beta$ cells suggesting the presence of an alternative regulatory process.

Because targeting of PP-2B by AKAP79 results in an inhibition of PP-2B catalytic activity (18, 20, 35), we speculate that PKA may affect PP-2B activity by changes in PP-2B targeting to AKAP79. AKAP79 inhibition of PP-2B would explain our finding that expression of AKAP79 results in lower PP-2B cellular activity. AKAP targeting of PKA and PP-2B also occurs in native $\beta$ cells confirmed by our finding AKAP150, the rat homologue of AKAP79, in pancreatic $\beta$ cells and showing colocalization of a portion of PKA and PP-2B in these cells. Furthermore, it is possible that PKA activation may alter the activity or distribution of PP-2B. Previously, Dell'Aqua *et al.* (35) found that PKA and PKC phosphorylate AKAP79 resulting in an increase in AKAP79 in the cell soluble fraction. Our data confirms this change in distribution of AKAP79 and 150. In addition, we show that expression of AKAP79 diminished the translocation of PP-2B following forskolin stimulation. At this time we do not know whether the movement of PP-2B or AKAP150 alters reversible phosphorylation events that affect insulin secretion. However, our data supports an effect of PKA on PP-2B that is partially regulated via a common targeting protein, AKAP 79/150.

Independent of the mechanism by which PKA affects PP-2B, our data suggests that the two enzymes are involved in an intracellular feedback loop whereby a kinase promotes the activation of a phosphatase resulting in reversible phosphorylation of specific proteins. The net effect of such a feedback loop would be a return to a basal state of phosphorylation, resetting the $\beta$ cell for additional stimulations. The reciprocal function of these enzymes will ultimately affect shared substrates including proteins involved in endocytosis (46), desensitization of channels (25, 47) or other mechanisms involved in regulating insulin secretion (37, 48). Disrupting the equilibrium between the phosphorylation and dephosphorylation of these substrates could result in abnormal insulin secretion, which may explain why insulin secretion was lower in the AKAP79 expressing $\beta$ cells. For example, development of Alzheimer's disease is associated with increased phosphorylation of Tau resulting from altered targeting of PKA and PP-2B by AKAP79 (49). AKAP targeting of PKA and PP-2B in $\beta$ cells represents a mechanism for regulating insulin secretion through balanced reversible phosphorylation of specific substrates.

In summary, we have found that insulin secretion can be regulated by the reversible phosphorylation of $\beta$ cell proteins through the targeted effects of PKA and PP-2B.

## Acknowledgments

The authors would like to thank Dr. John Rabkin for the donation of FK506, Max Hallin for technical assistance with the RIAs on this project, and Audra Norris and Madhavi Murty for assistance with the manuscript preparation.

## References

1. **Prentki M, Matschinsky F** 1987 Ca2+, cAMP and phopholipid-derivied messengers in coupling mechanisms of insulin secretion. Physiol Rev 67:1185–1248
2. **Lester LB, Langeberg LK, Scott JD** 1997 Anchoring of protein kinase A facilitates hormone-mediated insulin secretion. Proc Natl Acad Sci USA 94:14942–14947
3. **Widmann C, Burki E, Dolci W, Thorens B** 1994 Signal transduction by the cloned glucagon-like peptide-1 receptor: comparison with signaling by the endogenous receptors of beta cell lines. Mol Pharmacol 45:1029–1035
4. **Leech CA, Holz GG, Habener JF** 1996 Signal transduction of PACAP and GLP-1 in pancreatic beta cells. Ann NY Acad Sci 805:81–93
5. **Gromada J, Dissing S, Bokvist K, Renstrom E, Frokjaer-Jensen J, Wulff BS, Rorsman P** 1995 Glucagon-like peptide 1 increases cytoplasmic calcium in insulin secreting betaTC3-cells by enhancement of intracellular calcium mobilization. Diabetes 44:767–774
6. **Thorens B, Deriaz N, Bosco D, De Vos A, Pipeleers D, Schuit F, Mecla P, Porret A** 1996 Protein kinase A-dependent phosphorylation of GLUT2 in pancreatic $\beta$ cells. J Biol Chem 271:8075–8081
7. **Gromada J, Ding W-G, Barg S, Renstrom E, Rorsman P** 1997 Multisite regulation of insulin secretion by cAMP-increasing agonists: evidence that glucagon-like peptide 1 and glucagon act via distinct receptors. Pflugers Arch 434:515–524
8. **Leach KL** 1998 Protein kinases and phosphatases in cellular signaling. In: Conn P (ed) Handbook of Physiology-Cellular Endocrinology. Oxford University Press, Inc., pp 225–253

9. **Ashcroft SJ** 1994 Protein phosphorylation and beta-cell function. Diabetologia 37:S21–S29
10. **Jones PM, Persaud SJ** 1998 Protein kinases, protein phosphorylation and the regulation of insulin secretion from pancreatic beta-cells. Endocr Rev 19:429–461
11. **Lang J** 1999 Molecular mechansims and regulation of insulin exocytosis as a paradigm of endocrine secretion. Eur J Biochem 259:3–17
12. **Ammala C, Eliasson L, Bokvist K, Berggren P-O, Honkanen RE, Sjoholm A, Rorsman P** 1994 Activation of protein kinases and inhibition of protein phosphatases play a central role in the regulation of exocytosis in mouse pancreatic $\beta$ cells. Proc Natl Acad Sci USA 91:4343–4347
13. **Ebihara K, Fukunaga K, Matsumoto K, Shichiri M, Miyamoto E** 1996 Cyclosporin A stimulation of glucose-induced insulin secretion in MIN6 cells. Endocrinology 137:5255–5263
14. **Robertson RP** 1986 Cyclosporin-induced inhibition of insulin secretion in isolated rat islets and HIT cells. Diabetes 35:1016–1019
15. **Moxy-Mims MM, Kay C, Light JA, Kher KK** 1998 Increased incidence of insulin-dependent diabetes mellitus in pediatric renal transplant patients receiving tacrolimus (FK506). Transplantation 65:617–619
16. **Marks B, McMahon HT** 1998 Calcium triggers calcineurin-depdendent synaptic vesicle recycling in mammalian nerve terminals. Curr Biol 8:740–749
17. **Carr DW, Stofko-Hahn RE, Fraser IDC, Cone RD, Scott JD** 1992 Localization of the cAMP-dependent protein kinase to the postsynaptic densities by A-kinase anchoring proteins: characterization of AKAP79. J Biol Chem 24:16816–16823
18. **Coghlan VM, Perrino BA, Howard M, Langberg LK, Hicks JB, Gallatin WM, Scott JD** 1995 Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. Science 267:108–112
19. **Faux MC, Scott JD** 1997 Regulation of the AKAP79-protein kinase C interaction by Ca2+/calmodulin. J Biol Chem 272:17038–17044
20. **Kashishian A, Howard M, Loh C, Gallatin WM, Hoekstra MF, Lai Y** 1998 AKAP79 inhibits calcineurin through a site distinct from the immunophilin-binding region. J Biol Chem 273:27412–27419
21. **Taigen T, DeWindt LJ, Lin HW, Molkentin JD** 2000 Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy. Proc Natl Acad Sci USA 97:1196–1201
22. **Shibata A, Ludvigsen CW, Naber SP, McDaniel ML, Lacy PE** 1976 Standardization of a digestion-filtration method for isolation of pancreatic islets. Diabetes 25:667–672
23. **Corbin JD, Reimann EM** 1974 A filter assay for determining protein kinase activity. Methods Enzymol 38:287–294
24. **Blumenthal DK, Takio K, Hansen RS, Krebs EG** 1986 Dephosphorylation of cAMP-dependent protein kinase regulatory subunit (type II) by calmodulin-dependent protein phosphatase. J Biol Chem 261:8140–8145
25. **Yakel JL** 1997 Calcineurin regulation of synaptic function: from ion channels to transmitter release and gene transcription. Trends Pharmacol Sci 18:124–134
26. **Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH** 1994 RAFT1 (rapamycin and FKBP12 target): a mammalian protein that binds to FKBP 12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell 78:35–43
27. **Brown EJ, Albers MW, Shin T, Ichikawa K, Keith CT, Lane WS, Schreiber SL** 1994 A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature 369:756–758
28. **Bierer BE, Mattila PS, Standaert RF, Herzenberg LA, Burakolff SJ, Crabtree G, Schreiber SL** 1990 Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. Proc Natl Acad Sci USA 87:9231–9235
29. **Fruman DA, Wood MA, Gjertson CK, Katz HR, Burakoff SJ, Bierer BE** 1995 FK506 binding protein 12 mediates sensitivity to both FK506 and rapamycin in murine mast cells. Eur J Immunol 25:563–571
30. **Sjoholm A** 1994 Inhibitory effects of cyclosporin A on rat insulinoma cell proliferation, polyamine content and insulin secretion. Mol Cel Endocrinol 99:21–24
31. **Wilson JR, Ludowyke RI, Biden TJ** 1998 Nutrient stimulation results in a rapid Ca2+-dependent threonine phosphorylation of myosin heavy chain in rat pancreatic islets and RINm5F cells. J Biol Chem 273:22729–22737
32. **Matsumoto K, Ebihara K, Yamamoto H, Tabuchi H, Fukunaga K, Yasuami M, Ohkubo H, Shichiri M, Miyamoto E** 1999 Cloning from insulinoma cells of synapsin I associated with insulin secretory granules. J Biol Chem 274:2053–2059
33. **Krueger KA, Ings EI, Brun AM, Landt M, Easom RA** 1999 Site-specific phosphorylation of synapsin I by Ca2+/calmodulin-dependent protein kinase II in pancreatic beta TC3 Cells. Diabetes 48:499–506
34. **Scott JD, Fischer EH, Takio K, DeMaille JB, Krebs EG** 1985 Amino acid sequence of the heat-stable inhibitor of the cAMP-dependent protein kinase from rabbit skeletal muscle. Proc Natl Acad Sci USA 82:5732–5736
35. **Dell'Acqua ML, Faux MC, Thorburn J, Thorburn A, Scott JD** 1998 Membrane targeting sequences on AKAP79 binds phosphatidylinositol-4,5-bisphosphate. EMBO J 17:2246–2260
36. **Carr DW, Hausken ZE, Fraser IDC, Stofko-Hahn RE, Scott JD** 1992 Association of the type II cAMP-dependent protein kinase with a human thyroid RII-anchoring protein, cloning and characterization of the RII-binding domain. J Biol Chem 267:13376–13382
37. **Redmon Jb, Olson LK, Armstrong MB, Greene MJ, Robertson RP** 1996 Effects of tacrolimus (FK506) on human insulin gene expression, insulin mRNA levels and insulin secretion in HIT-T15 cells. J Clin Invest 98:2786–2793
38. **Seijffers R, Ben-David O, Cohen Y, Karasik A, Berezin M, Newgard CB, Ferber S** 1999 Increase in PDX-1 levels suppresses insulin gene expression in RIN 1046–38 cells. Endocrinology 140:3311–3317
39. **Fraser IDC, Tavalin SJ, Lester LB, Langeberg LK, Westphal AM, Dean RA, Marion NV, Scott JD** 1998 A novel lipid-anchored A-kinase anchoring protein facilitates cAMP-responsive membrane events. EMBO J 17:2261–2272
40. **Holz IV GG, Kuhtreiber Willem M, Habener Joel F** 1993 Pancreatic beta-cells are rendered glucose-competent by the insulinotropic hormone glucagon-like peptide-1(7–37). Nature 361:362–365
41. **Zaitsev SV, Efendic S, Arkhammar P, Bertorello AM, Berggren PO** 1995 Dissociation between changes in cytoplasmic free Ca2+ concentration and insulin secretion as evidenced from measurements in mouse single pancreatic islets. Proc Natl Acad Sci USA 92:9712–9716
42. **Liu J, Farmer JD, Lane WS, Friedman J, Weissman I, Schreiber SL** 1991 Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 66:807–815
43. **Shapiro AMJ, Lakey JRT, Ryan EA, Korbut GS, Toth E, Warnock GL, Kneteman NM, Rajotte R** 2000 Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 343:230–238
44. **Easom RA** 1999 CaM kinase II: a protein kinase with extraordinary talnets germane to insulin exocytosis. Diabetes 48:675–684
45. **Hashinoto Y, King M, Soderling TR** 1988 Regulatory interactions of calmodulin-binding proteins: phosphorylation of calcineurin by autophosphorylated Ca2+/calmodulin-dependent protein kinase II. Proc Natl Acad Sci USA 85:7001–7005
46. **Liu JP, Sim ATR, Robinson PJ** 1994 Calcineurin inhibition of dynamin I GTPase activity coupled to nerve terminal depolarization. Science 265:970–973
47. **Khiroug L, Sokolova E, Giniatullin R, Afzalov R, Nistri A** 1998 Recovery from desensitization of neuronal nicotinic acetylcholine receptors of rat chromaffin cells is modulated by intracellular calcium through distinct second messengers. J Neurosci 18:2458–2466
48. **Drucker DJ, Philippe J, Mojsov S, Chick WL, Habener JF** 1987 Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. Proc Natl Acad Sci USA 84:3434–3438
49. **Jicha GA, Weaver C, Lane E, Vianna C, Kress Y, Rockwood J, Davies P** 1999 cAMP-dependent protein kinase phosphorylations on tau in Alzheimer's disease. J Neurosci 19:7486–7494