# Site-Specific Phosphorylation of Synapsin I by $Ca^{2+}$/Calmodulin-Dependent Protein Kinase II in Pancreatic βTC3 Cells

## Synapsin I Is Not Associated With Insulin Secretory Granules

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**Increasing evidence supports a physiological role of $Ca^{2+}$/calmodulin-dependent protein kinase II (CaM kinase II) in the secretion of insulin from the pancreatic β-cell, but the precise sites of action are not known. A role of this enzyme in neuroexocytosis is implicated by its phosphorylation of a vesicle-associated protein, synapsin I. Because of emerging similarities to the neuron with respect to exocytotic mechanisms, the expression and phosphorylation of synapsin I in the β-cell have been studied. Synapsin I expression in clonal mouse β-cells (βTC3) and primary rat islet β-cells was initially confirmed by immunoblot analysis. By immunoprecipitation, in situ phosphorylation of synapsin I was induced in permeabilized βTC3 cells within a $Ca^{2+}$ concentration range shown to activate endogenous CaM kinase II under identical conditions. Proteolytic digests of these immunoprecipitates revealed that calcium primarily induced the increased phosphorylation of sites identified as CaM kinase II–specific and distinct from protein kinase A–specific sites. Immunofluorescence and immunogold electron microscopy verified synapsin I expression in βTC3 cells and pancreatic slices but demonstrated little if any colocalization of synapsin I with insulin-containing dense core granules. Thus, although this study establishes that synapsin I is a substrate for CaM kinase II in the pancreatic β-cell, this event appears not to be important for the mobilization of insulin granules. *Diabetes* 48:499–506, 1999**

The reversible phosphorylation of endogenous proteins as a result of glucose-induced elevation in cytosolic $Ca^{2+}$ has long been proposed to be an important step in the regulation of insulin secretion from the pancreatic β-cell (1–3). Concordantly, numerous $Ca^{2+}$-dependent protein kinases (4–6) and a $Ca^{2+}$-dependent protein phosphatase, calcineurin (7), have been identified in various preparations of β-cells, but little is known about their endogenous substrates or function. Evidence has recently accumulated that one of these kinases, the multifunctional $Ca^{2+}$/calmodulin-dependent protein kinase II (CaM kinase II), may play an important role in insulin secretion. Glucose activates CaM kinase II in isolated rat islets in a concentration-dependent manner (8) that temporally correlates with the initial and sustained phases of insulin secretion (9). Other insulin secretagogues that promote, in part, the mobilization of intracellular $Ca^{2+}$ also activate CaM kinase II (10). Although CaM kinase II is an attractive candidate for the cellular events associated with exocytosis in the β-cell, a deeper understanding of its physiological role can only be attained subsequent to the identification of its endogenous substrates.

Recent advances to understand the molecular mechanism of neuroexocytosis implicate a role for CaM kinase II. A number of proteins featured in the soluble N-ethylmaleimide–sensitive fusion protein attachment protein (SNAP)-receptor hypothesis of exocytosis (11), vesicle-associated membrane protein (synaptobrevin), α-SNAP, SNAP-25, N-ethylmaleimide-sensitive fusion protein, and synaptotagmin, are phosphorylated by CaM kinase II in vitro (12–15), although the physiological significance of such events, or whether they actually occur in vivo, is not clear. A functional involvement of CaM kinase II is, however, supported by its phosphorylation of a prominent neuronal protein, synapsin I, a process that is facilitated by their mutual interaction on the surface of the synaptic vesicle (16). Synapsin I phosphorylated by CaM kinase II has a decreased affinity for vesicle phospholipids (17) and a compromised ability to bind and bundle actin (18,19). Thus, this event has been proposed as a mechanism whereby synaptic vesicles are released from microfilament tethers and made available for migration to and fusion with the presynaptic membrane (13).

Functional and phenotypic similarities are apparent between neurons and endocrine β-cells. Both cell types synthesize large dense-core peptide-containing granules as well

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BSA, bovine serum albumin; CaM kinase II, $Ca^{2+}$/calmodulin-dependent protein kinase II; Ig, immunoglobulin; MAP-2, microtubule-associated protein-2; PBS, phosphate-buffered saline; PKA, protein kinase A; SNAP, soluble N-ethylmaleimide–sensitive fusion protein attachment protein.

as smaller neurotransmitter-containing vesicles, although their abundance in the different cells is reciprocal (20). The fundamental principles of granule fusion described by the SNARE hypothesis hold for all mammalian cells (21,22), but it is of interest that the β-cell expresses many neuron-like isoforms of the prominent SNARE proteins (23,24), supporting the contention that common mechanisms of regulated secretion occur in these cells. Taken a step further, the β-cell was also shown recently to express the microtubule-associated protein-2 (MAP-2) (25), previously thought to be largely limited to neurons (26), and a synapsin I–like protein (27). Significantly, MAP-2 was established as a substrate for CaM kinase II in β-cells (25). This information has therefore prompted the present study to document the expression of synapsin I in β-cells and to assess the potential of this protein to serve as a substrate for CaM kinase II.

## RESEARCH DESIGN AND METHODS

**Materials.** RPMI 1640, glutamine, antibiotics, trypsin/EDTA, and fetal bovine serum were purchased from Life Technologies (Gaithersburg, MD). Protein A-Sepharose, endoproteinase Glu-C (*Staphylococcus aureus* V8), and α-hemolysin (*S. aureus* α-toxin) were purchased from Sigma (St. Louis, MO). Ribonuclease A and trypsin treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) were acquired from Worthington Biochemical (Freehold, NJ). [γ-$^{32}$P]ATP was purchased from Du Pont–NEN (Boston, MA). Monoclonal anti-synapsin I (clone 223) was purchased from StressGen Biotechnologies (Victoria, British Columbia, Canada). Anti-synapsin I polyclonal antibody was raised against rat brain synapsin I prepared by the method of Bennett et al. (28); the resulting antisera were purified to an immunoglobulin (Ig)G fraction enriched in anti-synapsin I by chromatography on synapsin I–agarose. Mouse recombinant CaM kinase IIα was provided by Dr. Roger Colbran (Vanderbilt University Medical Center, Nashville, TN). The catalytic subunit of bovine heart cAMP-dependent protein kinase was donated by Dr. Ben Harris (UNTHSC, Fort Worth, TX). All other chemicals were of the finest reagent grade available.

**Cell culture and permeabilization.** Cell (βTC3; obtained from Dr. Shimon Efrat, Albert Einstein College of Medicine, New York, NY) culture and permeabilization (α-hemolysin, 125–200 U · $10^6$ cell$^{-1}$ · 0.1 ml$^{-1}$) were performed as previously described (25). Free $Ca^{2+}$ concentrations in incubation buffers were determined using a $Ca^{2+}$-electrode (Orion, Beverly, MA) calibrated against known standards as described by Bers (29).

**Immunoblot analysis.** Immunoblot analyses were performed on nitrocellulose membranes using an ECL Western Blotting Analysis System (Amersham, Amersham, U.K.). Primary and secondary antibody incubations were performed for 1 h each at 25°C.

**Synapsin I phosphorylation and immunoprecipitation.** Permeabilized βTC3 cells (~2 × $10^6$ per condition) were preincubated at 37°C for 5 min in permeabilization buffer (20 mmol/l HEPES, pH 7.0, 140 mmol/l potassium glutamate, 5 mmol/l NaCl, 4 mmol/l $MgSO_4$, 1 mmol/l EGTA, 300 μmol/l $Na_2ATP$) containing 0.05 μmol/l free $Ca^{2+}$. Cells were then pelleted and resuspended in 200 μl of fresh permeabilization buffer containing either 0.05 or 5 μmol/l $Ca^{2+}$ and 300 μmol/l [γ-$^{32}$P]ATP (specific activity 0.333 Ci/mmol) and were incubated at 37°C for the indicated times. Phosphate incorporation into synapsin I was analyzed by immunoprecipitation procedures as described by Krueger et al. (25), except that SDS-PAGE was performed on 7.5% gels. In vitro phosphorylation of synapsin I using the purified components of synapsin I, cAMP kinase catalytic subunit, or CaM kinase IIα was performed under conditions defined previously (25).

**Phosphopeptide mapping of synapsin I after *S. aureus* V8 digestion.** Partial proteolysis with *S. aureus* V8 protease of in vitro or in situ $^{32}$P-labeled synapsin I was performed as described elsewhere (30) with the following modifications: the excised synapsin I band was incubated at room temperature for 30 min in 125 mmol/l Tris-HCl buffer (pH 6.8) containing 0.1% SDS, placed into wells of a 15% polyacrylamide gel, and overlaid with the same buffer supplemented with 20% glycerol and 4 μg *S. aureus* V8. Subsequent to autoradiography, separation by electrophoresis was conducted at 100 V for 20 min, interrupted for 30 min to allow cleavage, and continued at 200 V.

**Two-dimensional tryptic phosphopeptide mapping of synapsin I.** For phosphopeptide mapping, $^{32}$P-synapsin I was eluted from SDS polyacrylamide gels and subjected to tryptic digestion as described elsewhere (25). Two-dimensional separation of phosphopeptides by electrophoresis and chromatography was performed on an HTLE 7000 thin-layer electrophoresis apparatus (C.B.S., La Jolla, CA) as previously described (31), except that electrophoresis was conducted at 1.3 kV for 40 min and chromatography for 14 h using a phosphochromatography buffer (37.5% *n*-butanol, 25% pyridine, 7.5% glacial acetic acid vol/vol).

**Immunofluorescence.** βTC3 cells and freshly excised whole rat pancreases were fixed with methanol (–20°C) or 4% paraformaldehyde, respectively, by methods described previously (32,33). Blocking was conducted in phosphate-buffered saline (PBS; 10 mmol/l sodium phosphate, pH 7.2, 150 mmol/l NaCl) supplemented with 5% bovine serum albumin (BSA) and 5% normal serum from the secondary antibody host animal. Primary antibody (1:100) incubation continued overnight at 4°C followed by incubation with fluorochrome-conjugated secondary antibody (1:200) for 1 h at 37°C in the dark. Repeated washes (3 × 15 min) in PBS containing 0.1% Triton X-100 were conducted between steps. Visualization of slides was conducted on a Nikon Microphot FXA microscope.

**Electron microscopy.** Rat insulinoma tissue, propagated in NEDH rats (34), was immersion-fixed on ice for 4–6 h with 2% paraformaldehyde/0.2% glutaraldehyde in 0.1 mol/l PBS, pH 7.4, and then infiltrated with 2.3 mol/l sucrose overnight at 4°C to cryoprotect the tissue. Ultrathin sections (100 μm) were prepared at –95°C on a Reichert Ultracut S with a Reichert FCS cryochamber attachment and collected on Formvar/Carbon-coated glow- discharged 200-mesh nickel grids. Free aldehydes in the sections were quenched by incubation (3 × 5 min) with 0.05 mol/l glycine in 0.01 mol/l PBS, and then immunostaining was performed section side down on 20- to 30-μl droplets. Nonspecific sites were blocked by incubation (10 min) in 10 mmol/l sodium phosphate, 150 mmol/l NaCl, pH 7.4, 0.5% BSA, 0.1% gelatin, and 20 mmol/l $NaN_3$ containing 5% serum from the secondary antibody host animal. Incubation with the primary and secondary antibodies, as well as the gold complex, was conducted for 1 h each. Control grids were exposed to preimmune serum or ascites fluid as appropriate. Grids were rinsed (6 × 5 min) between each incubation step and (3 × 5 min) after the gold-complex incubation. Sections were finally fixed in 1–2% glutaraldehyde/PBS for 15–20 min, rinsed (2 × 5 min) in PBS, further rinsed in distilled water (5 × 5 min), and reembedded/stained in a 2% aqueous methyl cellulose (M-6385; Sigma) containing 0.2% uranyl acetate for 10 min. Grids were air dried and viewed on a Zeiss 910 electron microscope at 100 kV accelerating voltage. Images were recorded on Kodak SO-163 electron films and processed in a Mohr Pro 8 film/paper processor.

**Statistical analysis.** Data are expressed as means ± SE determined from at least three independent observations unless otherwise stated. Where indicated, statistical significance ($P < 0.05$) was assessed by an independent Student's *t* test (SAS Institute, Cary, NC).

## RESULTS

**Expression and phosphorylation of synapsin I in βTC3 cells and pancreatic islets.** Despite its prominent role in neuron function, synapsin I has been reported as either absent or expressed at only very low levels in the pancreatic β-cell (20). Only with the recent demonstration of a synapsin I–like protein in the clonal β-cell line, MIN-6 (27), has the synapse-specific role of this protein been challenged. In the current study, immunoblot analysis using an affinity-purified polyclonal anti-synapsin I antibody also confirmed the presence of synapsin I–like proteins ($M_r$ ~85,000) in βTC3 cells and extracts generated from isolated rat pancreatic islets. That this β-cell protein was authentic synapsin I was supported by *1*) the recognition of β-cell immunoprecipitates by a monoclonal anti-synapsin I antibody (Fig. 1*B*), *2*) its in situ phosphorylation induced by $Ca^{2+}$ (see below), and *3*) the similarity of its phosphopeptide map to that generated from purified synapsin I (see below).

Neuronal synapsin I possesses at least six distinct sites that are phosphorylated by different kinases: site 1 (Ser-9) is phosphorylated by protein kinase A (PKA) and CaM kinases I and IV; site 2 (Ser-566) and site 3 (Ser-603) are phosphorylated by CaM kinases II and IV (35); and sites 4, 5, and 6 (Ser-62, -67, and -549, respectively) are phosphorylated by mitogen-activated protein kinase (36). To determine whether synapsin I serves as a physiological substrate for CaM kinase II in the pancreatic β-cell, the ability of $Ca^{2+}$ to induce synapsin I phosphorylation in α-toxin–permeabilized βTC3 cells was assessed. Under these experimental conditions, the details of which have been described previously (25), activation of CaM kinase II can be achieved by the elevation of $Ca^{2+}$ from 0.05 to 5 μmol/l. In the current study, a similar manipulation

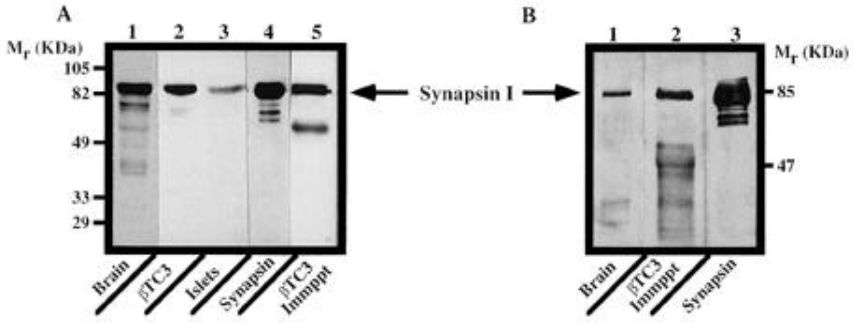


**FIG. 1. Expression of synapsin I in βTC3 cells and isolated rat pancreatic islets. Immunoblot analyses were performed using rabbit polyclonal (*A*) or mouse monoclonal (*B*) anti-synapsin antibodies. *A*: *Lane 1*, whole rat brain homogenate (15 µg protein); *lane 2*, βTC3 homogenate (20 µg protein); *lane 3*, isolated rat islets (20 µg protein); *lane 4*, purified rat brain synapsin I (1.3 µg protein); *lane 5*, rabbit anti-synapsin immunoprecipitate from βTC3 cells (lower band represents dissociated antibody). *B*: *Lane 1*, whole rat brain homogenate (20 µg protein); *lane 2*, rabbit anti-synapsin immunoprecipitate from βTC3 cells; *lane 3*, purified synapsin I (1.3 µg protein).**

significantly increased phosphate incorporation into synapsin I, as detected by immunoprecipitation and autoradiography. At 0.5 and 1 min, 5 µmol/l $Ca^{2+}$ induced 1.9- and 1.8-fold increases in phosphate incorporation into synapsin I relative to that observed under control conditions of 0.05 µmol/l $Ca^{2+}$ (Fig. 2*A*). Because this latter condition corresponds approximately to the intracellular $Ca^{2+}$ concentration expected in a resting β-cell, the modest phosphorylation observed under these conditions likely reflected the activity of basal kinase activities. Further analysis of the $Ca^{2+}$ dependency of synapsin I phosphorylation revealed that ion concentrations >0.5 µmol/l were necessary to induce significant synapsin I phosphorylation (Fig. 2*B*). At the maximally effective concentration, 5 µmol/l $Ca^{2+}$, $^{32}P_i$ incorporation was 208% of control (0.05 µmol/l $Ca^{2+}$), and half-maximal phosphorylation was achieved at ~1 µmol/l $Ca^{2+}$.

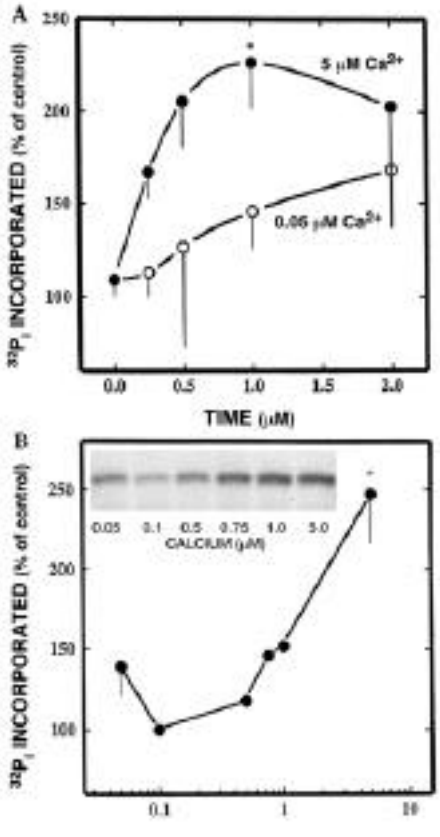


**FIG. 2. Time- and calcium-dependent phosphorylation of synapsin I in situ. Synapsin I was immunoprecipitated from βTC3 cells, and phosphate incorporation was quantitated by autoradiography and densitometry. *A*: Permeabilized βTC3 cells were incubated in buffers containing free $Ca^{2+}$ concentrations of 0.05 (○) or 5 (●) µmol/l at 37°C for the indicated times. **P* < 0.04 vs. time control (independent Student's *t* test). *B*: Permeabilized βTC3 cells were stimulated with increasing concentrations of calcium (0.05–5 µmol/l) at 37°C for 1 min. The inset displays an autoradiogram of immunoprecipitated synapsin I phosphorylated at the indicated $Ca^{2+}$ concentrations (µmol/l). **P* < 0.02 vs. 0.05 µmol/l $Ca^{2+}$ (independent Student's *t* test).**

**Identification of site-specific phosphorylation of synapsin I by SV8 digest and two-dimensional phosphopeptide mapping.** To confirm that the calcium-induced phosphorylation of synapsin I in βTC3 cells in situ was mediated by CaM kinase II, phosphopeptide mapping after limited proteolysis was performed. The cleavage of β-cell synapsin I with *S. aureus* V8 protease followed by one-dimensional separation by SDS-PAGE yielded two major phosphorylated products of ~30 and 10 kDa (Fig. 3, *lanes 3* and *4*). Based on the similarities with digests created from in vitro phosphorylation of rat brain synapsin I by purified enzymes (Fig. 3, *lanes 1* and *2*) and previously established criterion (30), these phosphoproducts harbor site 1 (10 kDa) and site 2 (30 kDa) targeted by PKA and CaM kinase II, respectively. In the presence of basal $Ca^{2+}$ (0.05 µmol/l), phosphorylation of β-cell synapsin I was primarily at site 1, while phosphorylation stimulated by 5 µmol/l $Ca^{2+}$ was observed in both sites, although most dra-

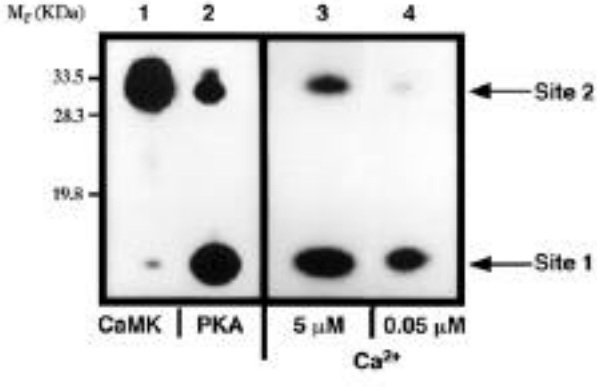


**FIG. 3. Phosphopeptide analyses of synapsin I phosphorylated in vitro and in situ after *S. aureus* V8 digestion. Rat brain synapsin I was phosphorylated by recombinant CaM kinase II or by the catalytic subunit of PKA (*lanes 1* and *2*, respectively), as described in METHODS. In situ phosphorylated synapsin I was immunoprecipitated from permeabilized βTC3 cells incubated for 1 min at 37°C with buffers containing free $Ca^{2+}$ concentrations of 0.05 (*lane 4*) and 5 (*lane 3*) µmol/l. Recovered synapsin was subjected to limited digestion by SV8 protease and to one-dimensional phosphopeptide mapping, as described in METHODS.**

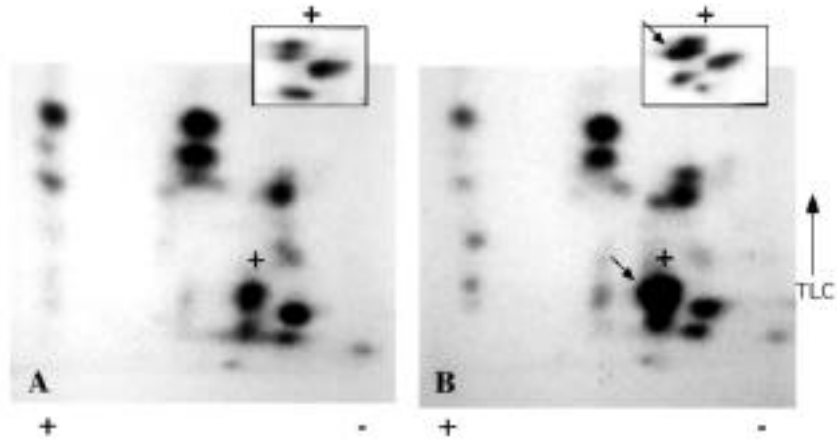


**FIG. 4. $Ca^{2+}$-induced phosphorylation of synapsin I in permeabilized βTC3 cells; two-dimensional tryptic phosphopeptide analysis. In situ phosphorylated synapsin I was immunoprecipitated from permeabilized βTC3 cells incubated with buffers containing free $Ca^{2+}$ concentrations of 0.05 (*A*) and 5 (*B*) µmol/l, subjected to tryptic digestion and to two-dimensional $^{32}$P-phosphopeptide mapping. Peptides were separated by electrophoresis in the horizontal dimension and ascending chromatography in the vertical dimension. Inset represents an enlargement of the lower portion of each of the maps indicated by +. Arrow (*B*) indicates phosphopeptide not apparent in control incubations. Each thin-layer plate was loaded with 265 cpm (Cerenkov).**

matically at site 2 (Fig. 3). Phosphate incorporation into site 2 was increased by 1.63-fold in comparison to the 1.21-fold increase in site 1, providing evidence that $Ca^{2+}$ induces the increased phosphorylation of a site distinct from that targeted by PKA. These results were similar to those observed previously in synaptosomes (30).

Two-dimensional tryptic phosphopeptide mapping was used to confirm that $Ca^{2+}$-dependent phosphorylation of synapsin I occurred at CaM kinase II–selective sites. For these experiments, immunoprecipitated synapsin I was subjected to trypsin digestion. Phosphopeptide maps of synapsin I recovered from cells incubated in 0.05 vs. 5 µmol/l $Ca^{2+}$ were very similar, with 6–8 prominent phosphorylation sites evident in both cases (Fig. 4). The elevation of $Ca^{2+}$, however, resulted in a marked enhancement in phosphate incorporation into a peptide(s), indicated by a plus sign (+) (Fig. 4*B* cf. Fig. 4*A*). Closer analysis of this area from duplicated experiments (illustrated in figure insets) revealed that this enhanced site actually represented the summation of three major phosphopeptides in contrast to the presence of two minor phosphopeptides in basal conditions (Fig. 4). Thus, one site (indicated by the arrow) is created under 5 µmol/l $Ca^{2+}$, and the phosphate incorporation into one of the two other sites was enhanced. Increased and decreased phosphorylation states of several other peptides in $Ca^{2+}$-stimulated cells were also observed but were quantitatively less significant (Fig. 4).

Further evidence for the identity of this major site targeted in $Ca^{2+}$-stimulated β-cells as a CaM kinase II–selective site was sought through comparison to maps generated from in vitro phosphorylation of neuronal synapsin I. $Ca^{2+}$-dependent phosphorylation of rat brain synapsin I by recombinant CaM kinase II yielded six major phosphopeptide species (Fig. 5*A*, peptides a–f), two of which (peptides d and e) comigrated with phosphopeptides recovered from β-cell synapsin I (peptides 1 and 2, Fig. 5*B* and *C*). Close comparison of these maps indicate that the peptide most responsive to $Ca^{2+}$ (labeled with the arrow in Figs. 4*B* and 5*B*) corresponds to the CaM kinase II site d in the in vitro map (Fig. 5*A*). The acidic phosphopeptides prominent in the in vitro maps, but barely visible in in situ maps (Fig. 5*A* and *B*, peptides a, b, and c), likely represent $NH_2$-terminal cyclized pyroglutamyl derivatives of peptides d, e, and f (Fig. 5*A*) as previously identified by Czernik et al. (35). Of the remaining phosphopeptides visible in in situ maps, three peptides, peptides 5, 8, and 9, were found to be contributed by the action of endogenous PKA based on their comigration with peptides generated when synapsin I was phosphorylated by the purified catalytic subunit of PKA (Fig. 6, peptides a, b, and d).

**Immunofluorescence and immunogold electron microscopy.** Immunocytochemistry was performed to examine the cellular distribution of synapsin I in pancreatic β-cells. Synapsin I was observed to be perinuclear and dispersed throughout the cytoplasm of βTC3 cells, consistent with its potential association with secretory machinery (Fig. 7*A* and *B*). This technique was further used to ascertain whether the expression of synapsin I was also characteristic of the primary β-cell.

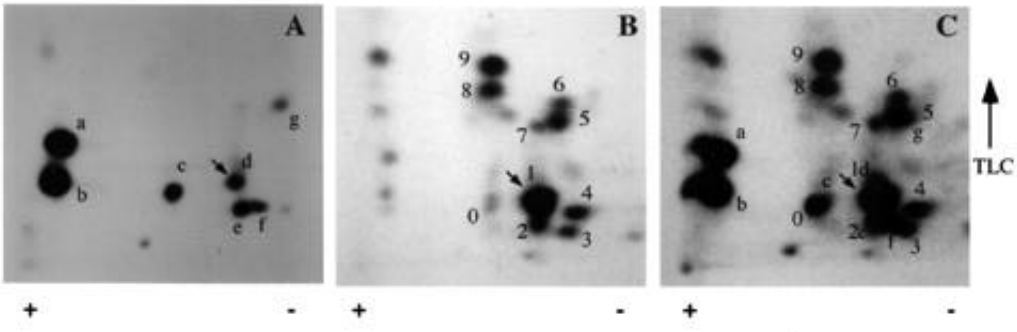


**FIG. 5. Identification of CaM kinase II–specific phosphorylation sites in synapsin I. Two-dimensional $^{32}$P-phosphopeptide maps were generated from rat brain synapsin phosphorylated by recombinant CaM kinase IIα (*A* and *C*) and synapsin immunoprecipitated from permeabilized βTC3 cells stimulated for 1 min at 37°C in the presence of 5 µmol/l $Ca^{2+}$ (*B* and *C*). *C*: A mix of in vitro and in situ phosphorylated synapsin I (*A* and *B*, respectively). Peptides phosphorylated by CaM kinase IIα in vitro (*A*) or phosphorylated in situ (*B*) are indicated by letters and numbers, respectively. The arrow indicates the peptide identified in Fig. 4 that comigrates with peptide *d* in the in vitro map. Cerenkov radioactivities loaded onto thin-layer plates were 2,000 (*A*), 265 (*B*), and 265 (*C*) cpm each.**

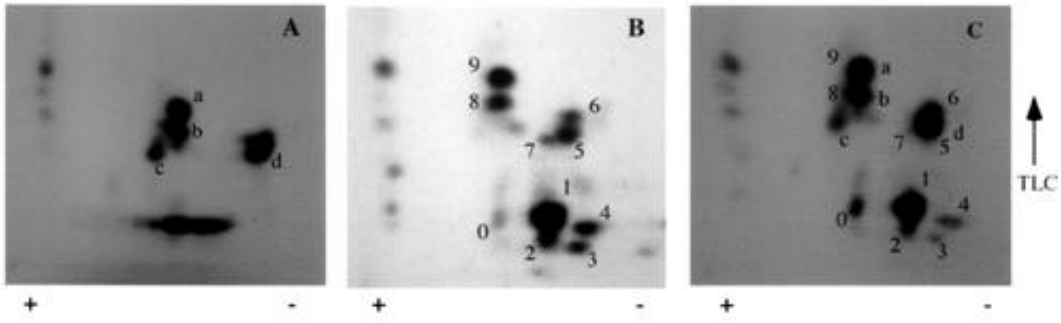


**FIG. 6. Identification of PKA-specific phosphorylation sites in synapsin I. Purified synapsin I phosphorylated by PKA (*A*) and in situ phosphorylated synapsin I immunoprecipitated from permeabilized βTC3 cells stimulated for 1 min at 37°C with buffers containing free $Ca^{2+}$ concentrations of 5 μmol/l (*B*) were digested with trypsin and subjected to two-dimensional [$^{32}$P]phosphopeptide mapping as described in METHODS. *C*: The resulting tryptic phosphopeptide map of the mix of *A* and *B*. Cerenkov counts per minute loaded for each sample were as follows: *A:* 2,000 cpm; *B:* 265 cpm; and *C:* 265 cpm each. Letters label in vitro–produced phosphopeptides (*A*), while numbers label in situ–generated phosphopeptides (*B*). The comigration map (*C*) is also labeled accordingly.**

Individual islets were identified in frozen sections of rat pancreas through immunofluorescence labeling with anti-insulin antibodies (Fig. 7*E*). Islets in subsequent slices were labeled specifically with anti-synapsin I antibody (Fig. 7*C*) in a manner that could be virtually eliminated by preabsorption of the antibody with purified rat brain synapsin I (Fig. 7*D*). Insulin immunoreactivity described a punctate pattern expected from the distribution of secretory granules. In contrast, however, immunolocalization of synapsin I appeared diffusely distributed in the cytoplasm of these cells. In double-labeling studies not shown, islet cells immunoreactive to anti-insulin or anti-glucagon antibodies expressed synapsin I, indicating a distribution throughout core β-cells as well as in mantle α-cells. Immunogold labeling performed in insulinoma tissue yielded additional evidence regarding synapsin I localization (Fig. 8). Figure 8*A* was representative of the pattern of synapsin I immunoreactivity frequently witnessed. Noteworthy was the routine observation of the grouping of synapsin I label, appar-

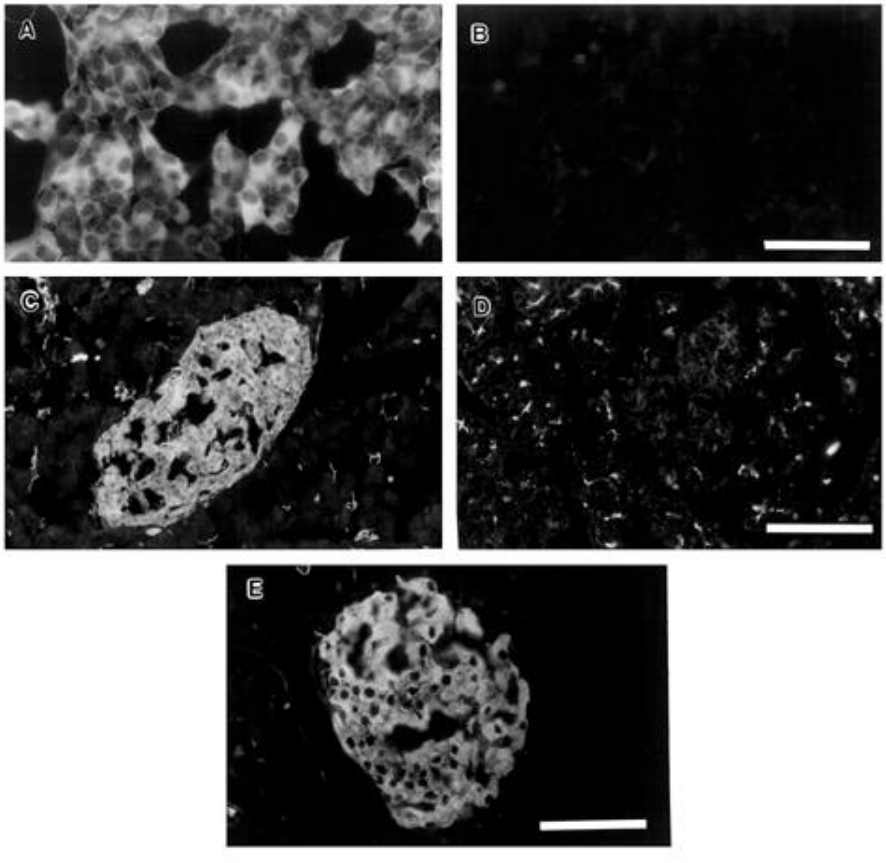


**FIG. 7. Synapsin I immunoreactivity in βTC3 cells and rat islets in frozen pancreas slices. Synapsin I was visualized by indirect fluorescence with a fluorescein-conjugated anti-rabbit antibody, while a rhodamine-conjugated anti-mouse antibody aided in the detection of insulin-expressing β-cells. *A* and *B:* βTC3 cells. *C–E:* Rat pancreases. Primary antibodies were synapsin I (*A*), normal rabbit IgG (*B*), synapsin I (*C*), synapsin I antibody preabsorbed with purified rat brain synapsin I (*D*), and insulin (*E*). Scale bar = 51 μm for *A* and *B*, 160 μm for *C* and *D*, and 80 μm for *E*.**

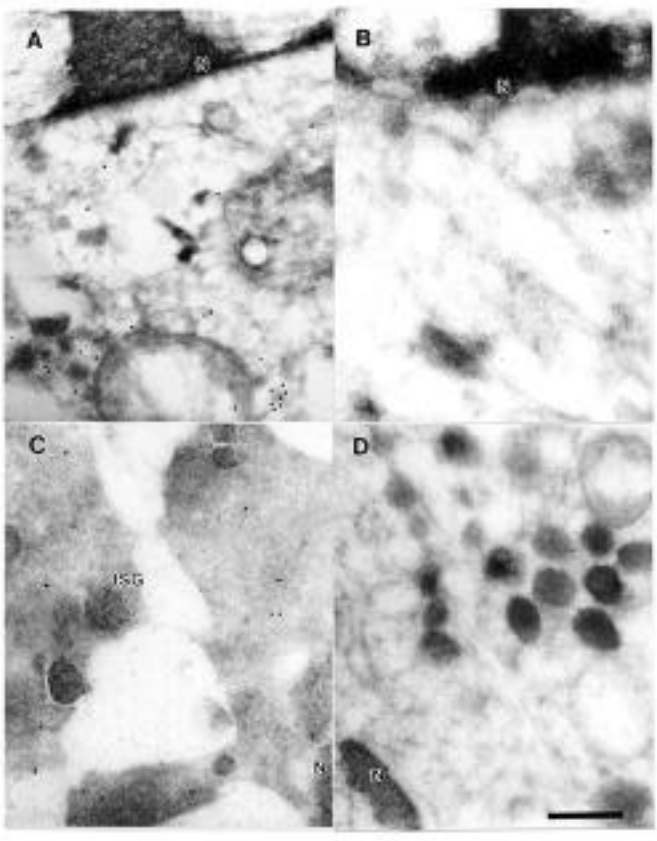


**FIG. 8. Localization of synapsin I and insulin immunoreactivity in rat insulinoma demonstrated by immunogold labeling. Cryosections of insulinoma were prepared and immunostained as described in METHODS. Anti-synapsin I was visualized using a biotin-conjugated secondary antibody/streptavidin-conjugated gold complex (15 nm). Anti-insulin was visualized by direct binding of gold-conjugated (5 nm) anti-mouse antibody. *A*: Synapsin I (1:100); *B*: normal rabbit IgG; *C*: synapsin I (1:200) and insulin (1:1,000); *D*: ascites fluid. The double labeling (*C*) indicates that synapsin I (larger gold particles, 15 nm) is localized to areas distinct from insulin (smaller gold particles, 5 nm). Scale bar = 0.4 μm.**

ently associated with vesicle-like organelles of a size inferior to the insulin secretory granule (lower right portion of Fig. 8*A*). Disparate labeling of synapsin I and insulin was also observed when isolated islets were visualized using similar methods (data not shown). Double-labeling studies indicated that while insulin was selectively associated with dense core granules, synapsin I demonstrated little if any colocalization with the same granules (Fig. 8*C*).

## DISCUSSION

Although a common origin for endocrine cells and neurons has been disproved (37), many of the mechanisms are conserved between the two cell types. In addition to the expression of neuron-like SNARE proteins, several other synaptic vesicle membrane proteins, synaptophysin, synaptobrevin, SV2, and rab3A, are expressed in endocrine pancreatic cells as well as other peptide-secreting endocrine cells (38–40). The current study documents the expression of the prominent neuronal protein, synapsin I, in cytoplasmic compartments of the pancreatic β-cell. Synapsin I was routinely detected as a doublet band in immunoblot and immunoprecipitation analyses of clonal β-cells or islets (not clear from figure illustrations) and, as such, was reminiscent of neuronal expression of splice variants synapsin I a/b (41). This doublet observation was in contrast, however, to the previous detection of a single "synapsin-like" protein ($M_r$ 84,000) in MIN-6 cells (27).

Importantly, synapsin I was also detected in primary islets (by immunoblot and immunofluorescence), eliminating the possibility that synapsin I expression in βTC3 cells was the result of phenotypic changes often characteristic of immortalized β-cells maintained in long-term culture (42). Although not pursued, it was noted that synapsin I was also detected in glucagon-expressing α-cells that constitute, in part, a population of cells forming a mantle around the core of β-cells. This may be significant, since α-cells, like β-cells, possess both dense core peptide granules as well as synaptic-like microvesicles, although the neurotransmitter content of the latter has not yet been determined (43).

A primary goal of this study was to determine whether synapsin I serves as a substrate for CaM kinase II in the pancreatic β-cell. Several pieces of evidence support this hypothesis and further suggest that this may be a physiologically relevant event. First, phosphate incorporation into synapsin I in permeabilized βTC3 cells occurred in response to concentrations of calcium required to induce the autophosphorylation and activation of CaM kinase II. In this respect, synapsin I phosphorylation closely correlated with the phosphorylation of MAP-2 at CaM kinase II–specific sites (25) and occurred within $Ca^{2+}$ concentrations required to support insulin secretion from permeabilized cells (44). Second, $Ca^{2+}$ induced the phosphorylation of sites identified as CaM kinase II–targeted sites based on two criteria. By one-dimensional SV8 digestion, the more significant phosphorylation induced by $Ca^{2+}$ occurred in the larger fragment corresponding to a COOH-terminal portion of synapsin I harboring CaM kinase II sites 2 and 3 (annotated as site 2 in the literature) (30). The modest phosphorylation observed in the smaller fragment (site 1) is likely attributable to the activation of a calcium/calmodulin-dependent adenylate cyclase (and possibly a cAMP phosphodiesterase) (45) with subsequent phosphorylation of an $NH_2$-terminal site through the activation of PKA. This result was reminiscent of $Ca^{2+}$-induced phosphorylation of MAP-2 at PKA sites observed in permeabilized β-cells (25). By two-dimensional phosphopeptide mapping, the differential phosphorylation induced by the elevation of $Ca^{2+}$ was accounted for primarily by the phosphorylation of a site (peptide 1) that corresponded with a major phosphopeptide (peptide d) targeted by recombinant kinase in vitro. This data is interpreted as validation of synapsin I as a substrate for CaM kinase II in the β-cell. These maps are inevitably complicated by other phosphorylation events. Of particular interest perhaps is that $Ca^{2+}$ induced the phosphorylation of a second site incorporated in the cluster termed peptide 1, indicating the possible presence of a second site targeted by CaM kinase II. Other observed differences could reflect subtle sequence differences between synapsins from these different sources or a differential presentation of substrate to kinase in in vivo versus in vitro conditions. Nevertheless, maps were similar to that determined previously for CaM kinase II phosphorylated synapsin I from rat and bovine brain, which located sites 2 and 3 in the lower half of the map (35). Finally, the evidence obtained that synapsin I is also a substrate for PKA in the β-cell is of interest in light of the necessary coordination of $Ca^{2+}$- and cAMP-dependent signaling pathways in the physiological maintenance of β-cell function (46).

Neither the function of synapsin I phosphorylation in the β-cell nor the significance of phosphorylation by CaM kinase II is clear. Previous studies in perifused rat islets have indicated that CaM kinase II may regulate secretory processes that are temporally, and possibly physically, removed from the exocytosis events at the plasma membrane (9). A suggestion that CaM kinase II functions to release insulin granules from cytoskeletal attachments (i.e., a reserve pool) thus seemed consistent with one model of the functional relationship of CaM kinase II, phosphosynapsin I, and neurotransmitter release proposed principally by Greengard et al. (13) and briefly described in the introduction. From the detection of a synapsin I–like protein in secretory granule fractions of MIN-6 cells, Matsumoto et al. (27) suggested that CaM kinase II–induced phosphorylation of this protein may similarly regulate insulin secretion in the β-cell. However, this contention is not supported from electron microscopy analyses in the current study in which immunogold labeling of synapsin 1 failed to colocalize with insulin-labeled secretory granules. The apparent grouping of gold particles with other smaller vesicular structures suggests rather that synapsin I may be important in the cell trafficking of non-insulin granules. These could include synaptic-like microvesicles that are considerably smaller (~50 nm) than insulin secretory granules (~70–200 nm) and that package and transport the neurotransmitter γ-aminobutyric acid (GABA) (20,47) or other undefined vesicular structures. It may be significant, therefore, that these synaptic-like microvesicles are released in conditions that favor CaM kinase II phosphorylation and insulin secretion (48).

The precise role of synapsin I in the neuron, where it is expressed to levels much greater than those observed in the β-cell, is still not understood. Actually, recent data obtained from a synapsin I/II double-knockout mouse model has discounted an essential function of synapsin in neuroexocytosis and has disputed the proposed function of synapsin I in the clustering and anchoring of synaptic vesicles to the microfilaments (49). The most evident defects in short-term measurements of synaptic plasticity in these animals suggest that synapsins may, in fact, be selectively required for the acceleration of synaptic vesicle traffic during repetitive stimulation at physiological frequencies (49). In this light, the physical and functional interaction with CaM kinase II makes sense, since the autophosphorylation and activation of this kinase is intricately designed to decode frequencies of $Ca^{2+}$ oscillations (50). Note that both synapsin I and II have recently been discerned, from crystal structure (51) and physical measurements (52), to be ATP-binding proteins that may function as phosphotransferases. Interestingly, with respect to regulated exocytosis, ATP binding to synapsin I, but not II, is $Ca^{2+}$ dependent (52). Whether phosphorylation of synapsin I by CaM kinase II modulates ATP binding has not yet been determined, but these novel properties may provide the necessary insights to elucidate the role of synapsin I phosphorylation in the pancreatic β-cell.

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