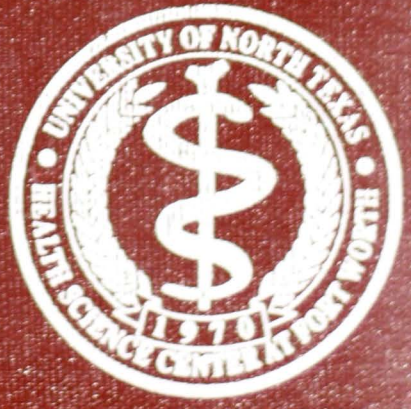
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Krueger, Kimberly A., The Phosphorylation of Endogenous Substrates by Calcium/Calmodulin-dependent Protein Kinase II in Pancreatic β-cells. Doctor of Philosophy (Biomedical Sciences), June, 1998, 165 pp., 35 illustrations, references, 259 titles.

Increasing evidence supports a physiological role for calcium/calmodulin-dependent protein kinase II (CaM kinase II) in the secretion of insulin from the pancreatic β-cell. While it has been previously demonstrated that CaM kinase II is activated by glucose in isolated rat islets implicating this enzyme in the secretion process, its cellular targets are unidentified. Potential candidates would likely exhibit strong binding to the enzyme, an association with the cytoskeleton, or an involvement in the secretion process. Based on these criteria, the following study represents as evaluation, *in situ* , of two proteins to function as substrates for CaM kinase II. Microtubule-associated protein, MAP-2 is one of the best substrates of CaM kinase II *in vitro* thought to be involved in secretion process. Synapsin I phosphorylation in the neuron by CaM kinase II is essential for neurotransmitter release. Unique to this study, both proteins were determined to be expressed in clonal mouse β-cells (βTC3) and primary rat islet β-cells. By immunoprecipitation, *in situ* phosphorylation of MAP-2 and synapsin I was induced in permeabilized βTC3 cells within a calcium range shown to activate endogenous CaM kinase II under identical conditions. Two-dimensional tryptic phosphopeptide mapping of both proteins revealed that sites phosphorylated by CaM kinase II *in vitro*, while distinct from sites phosphorylated by protein kinase A *in vitro*, were largely homologous to those sites phosphorylated *in situ* upon incubation of the βTC3 cells with increased free calcium. Immunofluorescence verified expression of both proteins in βTC3 cells and pancreatic slices, however, synapsin I exhibited little co-localization with insulin containing dense core granules as demonstrated

by immunogold electron microscopy. These data provide evidence that MAP-2 and synapsin I are phosphorylated by CaM kinase II in the pancreatic β-cell *in situ.* While the data suggest that synapsin I may not be involved in insulin secretion, an association with other known microvesicles of the β-cell, similarily secreted, may be possible. The phosphorylation of these CaM kinase II substrates may reveal an important intermediate step in the mediation of the glucose response in the pancreatic β-cell.

THE PHOSPHORYLATION OF ENDOGENOUS SUBSTRATES BY CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE II IN PANCREATIC β–CELLS

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DISSERTATION

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
University of North Texas Health Science Center at Fort Worth
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For the Degree of

DOCTOR OF PHILOSOPHY

By

Kimberly A. Krueger, B.S.
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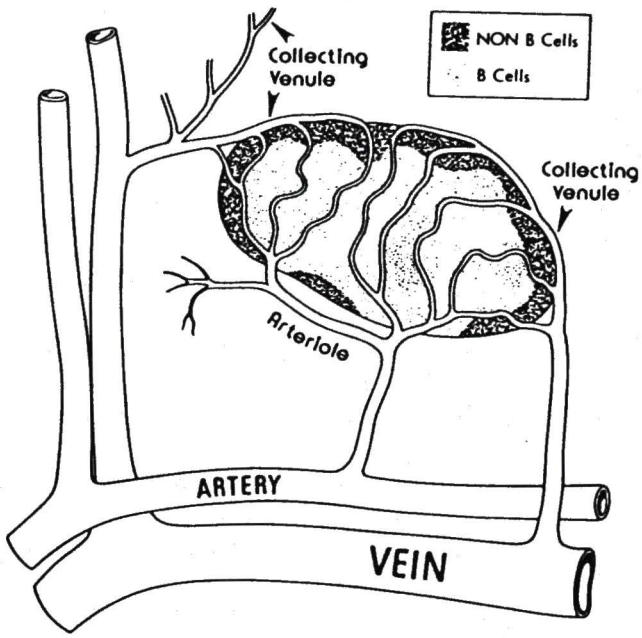
CHAPTER I

INTRODUCTION

Physiology of the Pancreatic β-cell --- The regulation of fuel metabolism involving a complex interplay between hormones, exogenous nutrients and endogenous substrates, is essential for ensuring an adequate supply of fuel for the body. The combined action of two key regulatory hormones, insulin and glucagon, are responsible for maintaining fuel homeostasis. The synthesis and secretion of insulin in response to changes in the metabolic state of the organism are the primary functions of the beta cells (β-cells) of the islets of Langerhans of the pancreas. The islets are dense clusters of peptide-secreting endocrine cells scattered throughout the pancreas, though most abundant in the tail of the pancreas (Howell, 1984). The insulin-secreting β-cells constitute approximately 85% of the islet endocrine cells and are surrounded by a mantle of other secretory cell types (Bonner-Weir, 1989). The bulk of the remaining cells are α-cells responsible for secreting glucagon released during times of acute glucose needs, such as during exercise or stress, effecting a rise in blood sugar. The few percent of islet cells left include δ-cells which secrete somatostatin known to inhibit insulin and glucagon release, and pancreatic polypeptide-secreting cells (Ashcroft *et al.* 1992).

The islets of Langerhans are maintained by an arterial blood supply that branches into a rich, intra-islet capillary bed, infusing the pancreatic endocrine and exocrine cells before emptying into the portal venous system (Fig. 1) (Bonner-Weir, 1988). The vascular arrangement is such that plasma containing freshly secreted insulin is carried to the α- and δ-cells, whereupon secreted glucagon and somatostatin may also enter the plasma stream.

FIG. 1. **Microvasculature of the islet.** Each arteriole penetrates the islet through gaps or discontinuities of the non-β-cell mantle. The capillaries pass first through the β-cell core and then the non-β-cell mantle. Taken from Bonner-Weir, S. 1989 Pancreatic Islets: Morphology, Organization, and Physiological Implication. In: Insulin Secretion, edited by Draznin, B., Melmed, S., LeRoith, D. New York: Alan R. Liss, Vol 1, pp. 1-11.

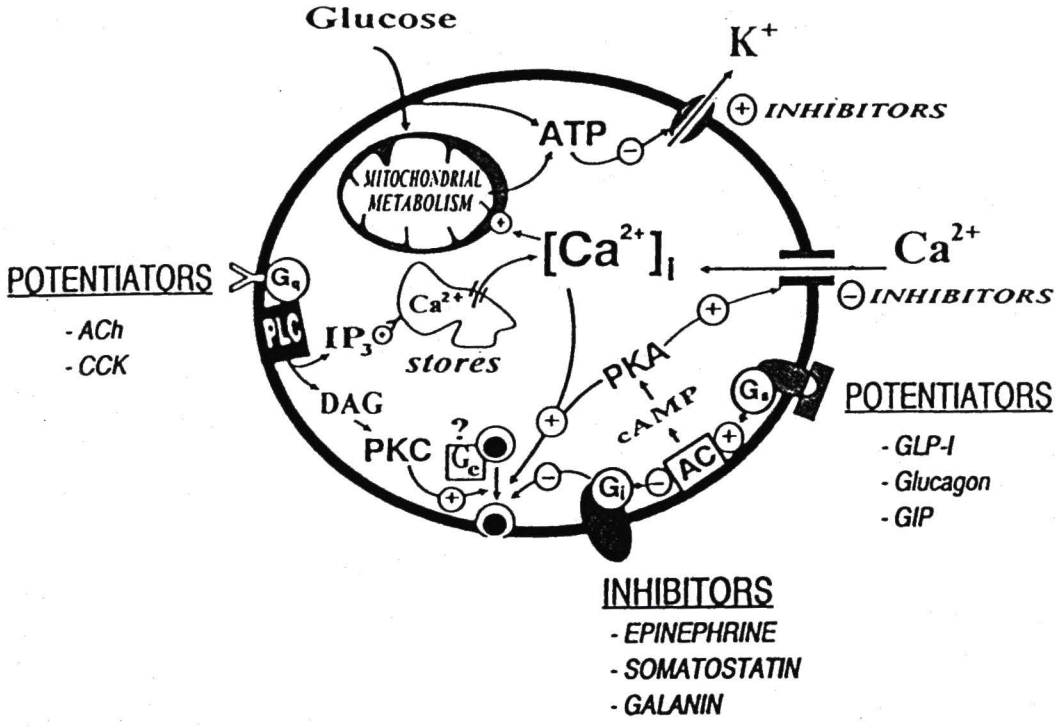

NON B Cells
B Cells
Collecting Venule
Collecting Venule
Arteriole
ARTERY
VEIN

This implies that locally secreted insulin by the β-cells exerts paracrine effects on α- and δ-cells, while the β-cells, which are stimulated by glucagon and inhibited by somatostatin, will be responsive to these hormones only after circulation through the body. The islets also receive innervation from sympathetic and parasympathetic nerves. Modulation of islet hormone release occurs via a variety of neurotransmitter receptor mechanisms; parasympathetic release of acetylcholine activates muscarinic receptors and activation of β-adrenergic receptors by norepinephrine (from sympathetic nerves) both result in potentiation of glucose-induced insulin secretion (Bergman *et al.* 1973; Ahren *et al.* 1987). Alternatively, activation of α-adrenergic receptors inhibits insulin secretion (Porte *et al.* 1966).

Thus, while the primary function of the β-cell is to regulate blood glucose levels via the appropriate secretion of insulin, β-cell activity can be modulated by a variety of other nutrients, hormones and neurotransmitters (Fig. 2). The β-cell acts as a fuel-sensing organ, integrating these neurohumoral and metabolic inputs into complex interactions of several signaling mechanisms in order to synthesize and secrete insulin according to the metabolic and physiologic needs of the organism. This unique feature of the β-cell to be responsive to such varied stimuli, is in contrast to other neurosecretory cells which are generally specialized, responding to only a few inputs.

Pathophysiology of Diabetes Mellitus --- It is glucose, however, that continues to be recognized as the key physiological regulator of β-cell activity. It not only directly regulates insulin synthesis and secretion, and is an essential metabolic fuel, but also moderates other major islet signals involved in metabolism, growth and maturation. Nonetheless, despite its importance, high plasma glucose levels such as those that characterize individuals with diabetes mellitus are pathogenic and can lead to serious micro- and macrovascular complications (Weir *et al.* 1994). Untreated, hyperglycemia can manifest into resulting

FIG. 2. **Scheme of signal transduction pathways in insulin secretion.** Acetylcholine (ACh), adenosine 5'-triphosphate (ATP), adenylate cyclase (AC), cholecystokinin (CCK), diacylglycerol (DAG), gastric inhibitory polypeptide (GIP), glucagonlike peptide 1 (GLP), inositol-1, 4, 5-triphosphate (IP_3), phospholipase C (PLC), protein kinase A (PKA), and protein kinase C (PKC). Taken from Wollheim, C.B., Lang, J. and Regassi, R. 1996 The exocytotic process of insulin secretion and its regulation by Ca^{2+} and G-proteins. Diabetes Rev. 4:276-297


Glucose
K+
INHIBITORS
ATP
MITOCHONDRIAL METABOLISM
[Ca2+]i
Ca2+
INHIBITORS
POTENTIATORS
- ACh
- CCK
Gq
PLC
IP3
Ca2+
stores
DAG
PKC
?
Gc
PKA
cAMP
AC
Gs
Gi
POTENTIATORS
- GLP-I
- Glucagon
- GIP
INHIBITORS
- EPINEPHRINE
- SOMATOSTATIN
- GALANIN

blindness, neuropathy, cardiovascular disease, nephropathy, acute metabolic disorders and premature mortality (Unger *et al.*1992).

Considered among the most common of chronic disorders, affecting from 5 to 10% of the adult population of the Western world, diabetes mellitus is a group of disorders characterized by abnormal glucose homeostasis resulting in hyperglycemia (Herman *et al.*1984). Moreover, because the disease is often early-on asymptomatic and subsequently undiagnosed, it's estimated that there is typically one undiagnosed case for each that is known. Diabetes mellitus is a genetic disorder in which the phenotypic expression of the disease is a result of environmental factors superimposed on genetic susceptibility. There are different forms of the disease expressed in terms of etiology, pathogenesis, historical development and response to treatment. These factors, including variability in its manifestations, establish that diabetes mellitus is not a single disease, but a syndrome; a genetically heterogeneous group of disorders that share glucose intolerance in common (Fajans *et al.*1978; Rotter *et al.*1992). Accordingly, establishing diagnostic criteria and a classification for diabetes, has been previously fraught with problems. As a result, in 1979, the National Diabetes Data Group and, in 1980, the World Health Organization (Harris *et al.*1979; WHO Study Group, 1980) recommended criteria currently used for the diagnosis of diabetes which describes three clinical classes: (1) characterization by fasting hyperglycemia or increased plasma glucose above a set limit during a glucose tolerance test; (2) impaired glucose tolerance during a glucose tolerance test exhibited by levels of plasma glucose between normal but below that defined as diabetes; (3) gestational diabetes. Diabetes mellitus itself is subdivided into four types that differ in etiology and pathogenesis. Type I or insulin-dependent diabetes mellitus (IDDM) is diagnosed in 10% of diabetic patients. The second type of diabetes, type II or noninsulin-dependent diabetes mellitus (NIDDM) is the most frequent form of diabetes in all parts of the world and present in 90% of diabetics in the Western world.

IDDM results from a chronic autoimmune destruction of the pancreatic β-cells thought to be initiated by genetically determined abnormal immune responses linked to histocompatibility locus antigens (HLA). This may impart increased susceptibility to β-cell damage by permitting interaction of an environmental factor with specific cell membrane antigens (Eisenbarth, 1986). Circulating islet cell antibodies (ICA), insulin autoantibodies (IAA) and antibodies to glutamic acid decarboxylase (GAD) aid in diagnosis but are transient and probably the result of the autoimmune process, rather than the cause (Atkinson *et al.* 1986). It is now apparent that IDDM gradually develops over many years with a variety of abnormalities in immune function and insulin release preceding the 'abrupt' appearance of the syndrome (Maclaren, 1988). IDDM alone is a major cause of cardiovascular disease and premature death and the leading cause of blindness, amputation and end-stage renal diseasse. It results in medical care expenditures of over $5 billion annually, with costs for diabetic patients over 10 times that for nondiabetic patients (Songer, 1990).

Classically IDDM, often termed juvenile-onset diabetes mellitus, occurs most commonly in childhood and adolescence affecting an estimated 50,000 new cases a year worldwide and 1 to 3 per 1000 children by the age of 20 years in Caucasian populations (Rewers, 1991). However, accumulating data suggest that IDDM is more common in older people than previously appreciated with approximately 50% of IDDM presented clinically after the age of 20 (Caillat-Zucman *et al.* 1992). In fact, although types I and II diabetes are clinically distinct, representing different disease processes, there is increasing pathophysiologic and epidemiologic evidence for heterogeneity within each of these groups and of overlap between these two major types of diabetes. Low or absent levels of endogenous insulin production until recently believed to be due to β-cell destruction, characterizing IDDM and distinct from NIDDM, is now recognized to be partly caused by functional impairment of the β-cell (McCulloch *et al.* 1988). Additionally, approximately 10

to 15% of diagnosed NIDDM patients were determined to be expressing islet cell and GAD antibodies of similar type as observed for type I diabetes (Hagopian *et al.* 1993).

However, near total β-cell loss, a prominent feature of type I diabetes, is not evident for NIDDM. As demonstrated by morphometric studies, type II diabetic patients have only lost at most 50% of their β-cells (Gepts *et al.* 1981). Because fasting hyperglycemia is not observed in experimental animals with 50% β-cell loss, additional factors, such as a dysfunction of the remaining β-cells, or concomitant insulin resistance, must contribute. Thus, NIDDM is characterized by defects in insulin secretion as well as insulin action. NIDDM, itself a heterogeneous disorder, is also thought to be initiated by a genetic predisposition challenged by environmental factors. Obesity, for example, is a frequent concomitant factor in NIDDM and often a predictor of diabetes (Colditz *et al.* 1990). Excessive caloric intake and the associated reduction in insulin-mediated glucose clearance (insulin resistance) are present in most (60 to 80%) affected NIDDM patients in Western societies (Defronzo, 1988). At the stage of impaired glucose tolerance, high levels of insulin circulate in response to the modest hyperglycemia, the impaired early insulin response and insulin resistance. When increasing insulin resistance demands an enhanced compensatory response that can not be met, a genetic defect in insulin secretion may then be revealed. This results in deteriorating glucose tolerance to levels diagnostic of NIDDM and increased fasting hyperglycemia. While obesity and pathogenic insulin resistance can exacerbate glucose intolerance and precipitate fasting hyperglycemia, they are not essential to the evolution of NIDDM (World Health Organization, 1985). Additionally, there is increasing support for the concept that the presence or absence of obesity may distinguish heterogeneous groups of NIDDM (Ohlson *et al.* 1987).

NIDDM is usually diagnosed in patients over the age of 40. Although more common in middle-aged individuals, NIDDM can occur at any age. Well defined abnormalities such as mutations in the insulin receptor, insulin genes, glucokinase or

mitochondrial genes are responsible for a small subset of NIDDM cases (Vionnet *et al.*1993). A subclass of NIDDM includes maturity-onset type diabetes of the young (MODY), characterized by onset before 25 years of age and autosomal dominant inheritance (Fajans, 1989). Mutations in the glucokinase gene have been associated with MODY (Owerbach *et al.*1993; Davies *et al.*1994).

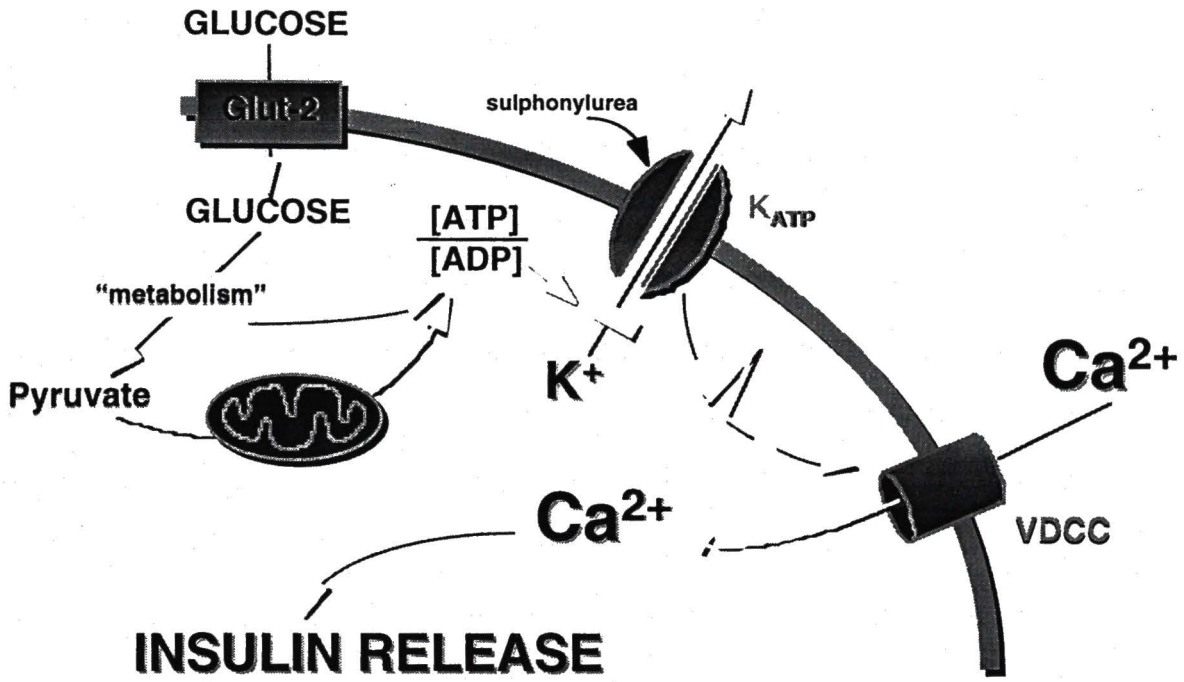
In animal models with selective impairment of insulin secretion, hyperglycemia is the dominant factor that maintains fasting insulin and acute insulin responses to nonglucose secretagogues such as arginine, in the normal range (Taborsky *et al.*1981). This is also observed in NIDDM patients which often appear to have normal or even elevated blood insulin levels. In order to evaluate insulin levels following a glucose challenge, any meaningful interpretation must take into account all participating variables such as current circulating glucose levels and glucose uptake by peripheral tissues. Thus, when NIDDM patients were adiposity (resistance) and glycemia matched, deficiencies in both basal and glucose-stimulated insulin secretion were unmasked (Gray *et al.*1994). Furthermore, examinations of pulsatile and oscillatory insulin secretion patterns between normal and NIDDM diabetic patients revealed abnormalities (O'Meara *et al.*1993). Not only was there a total absence of the acute or first-phase insulin response during the first 10 minutes after intravenous glucose administration, but second-phase insulin secretion was also decreased. As was the case for basal and second-phase insulin secretion, under matched conditions, the acute insulin response to a nonglucose stimulus was also determined to be reduced in diabetic subjects (Porte, 1991). These patterns of attenuated insulin secretion describe classic manifestations of NIDDM.

It appears therefore, that in type II diabetes mellitus, and to a certain degree in type I, there is a fundamental decrease in β-cell responsiveness to the prevailing plasma glucose level. Tight regulation of glucose levels center on the ability of the β-cell to secrete insulin in response to small incremental increases in glucose. Current treatment of diabetic patients

to modulate insulin levels by either administration of insulin injections or oral secretagogues that potentiate insulin secretion such as sulfonylureas or L-arginine, are unsatisfactory. Unable to address the causes of the disorder, these 'band-aid' treatments can only help to relieve the consequent hyperglycemia. Obviously due to the prevalence, incidence and debilitating complications associated with diabetes mellitus, extensive effort to elucidate the determinates of the disorder is well warranted. Because islet dysfunction plays a key role in the regulation of glucose homeostasis, detailed characterization of the biochemical mechanisms involved in insulin secretion will be necessary for the total comprehension of the etiology of NIDDM and to facilitate treatment and prevention of this disorder.

Model of Insulin Secretion --- The molecular mechanisms by which the β-cell senses and responds to physiological changes in plasma glucose levels have been the subject of intense investigation (Malaisse, 1995). The initial steps of the secretion process are considered to involve first, the facilitated diffusion of glucose on Glut-2 transporters, and then the subsequent production of glucose-6-phosphate catalyzed by glucokinase. The currently accepted model (Fig. 3) continues by proposing that the metabolism of glucose in the β-cell by glycolysis increases the ATP:ADP ratio which inhibits the ATP-sensitive K^+ channels (Ashcroft, 1988). The resulting depolarization of the plasma membrane causes the opening of the voltage-gated Ca^{2+} channels and associated influx of Ca^{2+} required for the fusion of insulin containing vesicles with the plasma membrane and the subsequent exocytotic event (Rajan *et al.*1990). Most recently, other additional metabolic coupling factors have been implicated that may influence the dose-dependency of glucose-induced insulin release (Gembal *et al.*1992). Uniquely in the β-cell, mitochondrial metabolic activation, as evidenced by an increase in β-cell NAD(P)H following glucose stimulation, precedes β-cell depolarization and Ca^{2+} elevation (Pralong *et al.*1994). ADP variations, rather than only

FIG. 3. **Mechanism of glucose-induced insulin secretion in the β-cell.** Glucose transporter (Glut-2) and voltage-gated calcium channel (VDCC).


GLUCOSE
Glut 2
sulphonylurea
GLUCOSE
[ATP]
[ADP]
K_{ATP}
"metabolism"
Pyruvate
K^+
Ca^{2+}
Ca^{2+}
VDCC
INSULIN RELEASE

ATP, affecting the ATP:ADP ratio is receiving more emphasis. Anaplerosis, or the replenishment of the Kreb's cycle with intermediates, appears essential for β-cell activation. A shift from fatty acid oxidation to esterification concomitant with the increased formation of malonyl-CoA and long chain acyl-CoA esters as additional metabolic coupling factors are also gaining recognition as influential components of the β-cell response to glucose (Prentki, 1996).

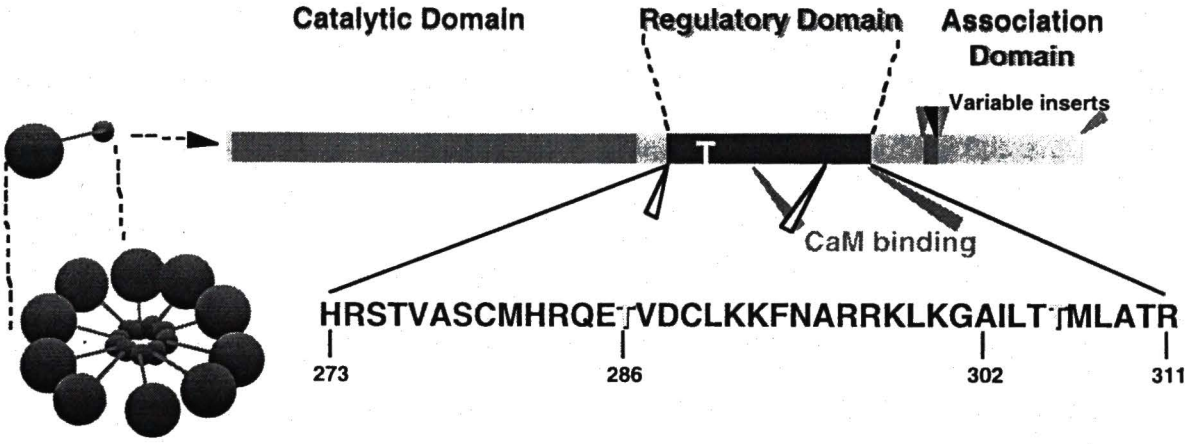
It is widely accepted that the highly regulated exocytosis of secretory vesicles requires an increase in intracellular Ca^{2+}. Indeed, increased intracellular Ca^{2+} is critical to glucose-induced insulin secretion (Jones *et al.* 1992; Jonas *et al.* 1994). While known to play a pivotal role in secretion, the sites of action of Ca^{2+} and the biochemical mechanisms by which increased cytosolic Ca^{2+} translates to secretion have yet to be determined. Possible hypotheses are that the action of Ca^{2+} in secretion is mediated by calmodulin-binding proteins and/or that Ca^{2+} regulates dynamic changes in the cytoskeleton (Trifaro *et al.* 1987). Studies on the mechanisms of hormone and neurotransmitter release have suggested that Ca^{2+} does indeed have an effect on the cytoskeleton during secretion and that the cytoskeleton and its associated proteins are intimately involved in the mediation of the secretion process (Trifaro *et al.* 1992; Trifaro *et al.* 1993). Because of increasing evidence that Ca^{2+}-dependent protein phosphorylation is also involved in the secretion process, it has been proposed that a cytoskeleton-associated Ca^{2+}/calmodulin-dependent protein kinase(s) may mediate the distal steps of secretion.

Ca^{2+}/Calmodulin-dependent Protein Kinase II --- A possible candidate is Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II). CaM kinase II, one of the most abundant Ca^{2+}-activated protein kinases, though predominantly expressed in neurons, is present in virtually all cell types (Rostas *et al.* 1992). CaM kinase II possesses a multifunctional property to selectively phosphorylate a broad number of substrates that contribute to the

mediation of a diverse array of cellular responses in response to elevated Ca^{2+} such as cell metabolism, neurotransmitter release and synthesis, Ca^{2+} homeostasis, cytoskeletal protein interactions, and postsynaptic responses; e.g. long term potentiation (Braun *et al.*1995; Hanson *et al.*1992a). Molecular cloning has identified four distinct classes of CaM kinase II (α, β, δ, γ) each encoded by a separate gene (Tobimatsu *et al.*1989). A holoenzyme, with an apparent subunit molecular mass ranging from 50 to 65 kDa, CaM kinase II is a multimer of 4 - 12 subunits, each containing a NH_2-terminal catalytic domain, a central regulatory (autoinhibitory and calmodulin-binding) domain, and a COOH-terminal association domain (Rostas *et al.*1992). The various subunit isoforms share approximately 80 - 90% homology within conserved catalytic and regulatory domains with the greatest divergence occurring within the association domain. For each isoform, 2 - 10 alternatively spliced variants, differing in insertions or deletions in the association domain, have been described concordantly in different tissues. This suggests that there may exist specific functional roles for each isoform (Mayer *et al.*1994; Edman *et al.*1994). Additionally, the association domain has been implicated in directing the subcellular localization of CaM kinase II (Srinivasan *et al.*1994; Zhou *et al.*1995) and in the assembly of the subunits to form the holoenzyme, resembling a hub-and-spoke pattern (Fig. 4) (Braun *et al.*1995).

CaM kinase II activity is regulated by Ca^{2+}/calmodulin (Ca^{2+}/CaM) binding and by autophosphorylation. Binding of Ca^{2+}/CaM relieves the inhibition exerted by the pseudosubstrate autoinhibitory domain on the catalytic domain, triggering autophosphorylation of $Thr^{286/287}$ (Hanson *et al.*1992b; Smith *et al.*1990). This increases the affinity of the kinase for calmodulin several hundredfold essentially 'trapping' calmodulin (Meyer *et al.*1992). This event, which temporally precedes the phosphorylation of exogenous substrates, results in a fully active kinase in the presence of Ca^{2+}/CaM and a partially active kinase in its absence (autonomous activity). Autophosphorylation occurs as

FIG. 4. **Structural properties of CaM kinase II.** The hub-and-spoke pattern of the holoenzyme in which each subunit contains a catalytic domain, an autoregulatory domain and an association domain. Taken from Braun, A. and Schulman, H. 1995 The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. Annu. Rev. Physiol. 57:417-445.


Catalytic Domain
Regulatory Domain
Association Domain
Variable inserts
T
CaM binding
HRSTVASCMHRQE VDCLKKFNARRKLKGAILT MLATR
273
286
302
311

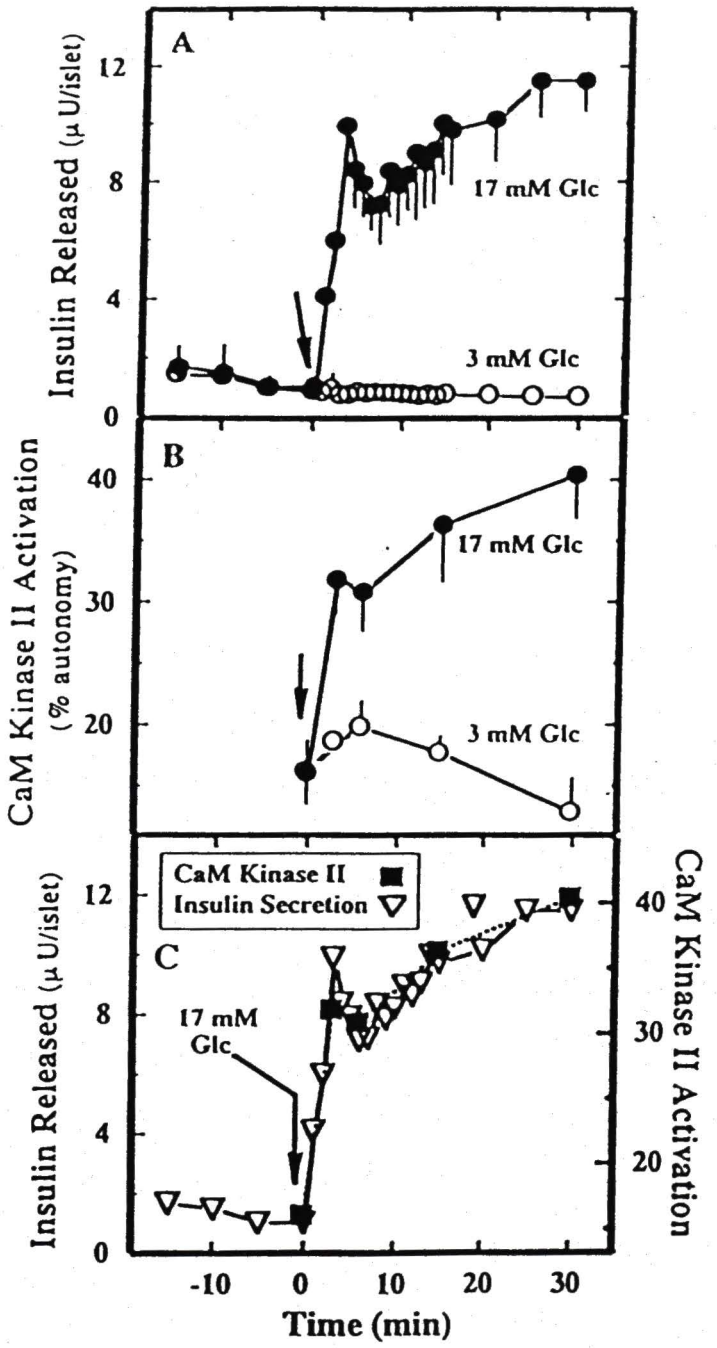
an intersubunit reaction between proximate subunits within the holoenzyme (Mukherji *et al.*1994). The ability of the Ca^{2+}/CaM-independent form to prolong the activity of CaM kinase II beyond the transient elevation of intracellular Ca^{2+}, has functional implication in memory models of the kinase and in detecting the frequency of potentially activating calcium spikes (De Koninck *et al.*1998; Lisman *et al.*1988). In the absence of Ca^{2+}/CaM, autophosphorylation at $Thr^{305/306}$ and Ser^{314} blocks Ca^{2+}/CaM binding, constraining the enzyme to a submaximal autonomous activity, and ultimately, inactivation (Colbran *et al.*1990; Hanson *et al.*1992b).

Research Indicating the Potential Role of CaM Kinase II in Insulin Secretion --- Due to the prevalence of CaM kinase II in the brain, initial investigations describing the properties of the kinase (i.e.; activity, cellular distribution and possible functional role) were conducted in neuronal tissue. However, as biochemical similarities between neurons and neuroendocrine cells have become increasingly evident (Wheeler *et al.*1996; Thomas-Reetz *et al.*1994), research performed in the neuron may be applicable to the pancreatic β-cell. Thus, results obtained from the neuron provide a basis of comparison and reveal possible clues regarding the role of CaM kinase II in the β-cell. Examination of the characteristics of CaM kinase II in the neuron and the β-cell defines the potential of this enzyme in the mediation of neurotransmitter release and insulin secretion, respectively. CaM kinase II autophosphorylation and subsequent kinase activity have been reported in intact cells in response to agonists that increase intracellular Ca^{2+} concentration (Jefferson *et al.*1991). Introduction of CaM kinase II into brain tissues was demonstrated to enhance the evoked release of neurotransmitter (Nichols *et al.*1990; Llinas *et al.*1991). Transgenic mice lacking the CaM kinase II α-subunit gene exhibit marked alterations in processes of presynaptic plasticity, are deficient in spatial learning and have abnormal fear response and aggressive behavior (Silva *et al.*1992; Chen *et al.*1994). While CaM kinase II has been traditionally

characterized from the brain, CaM kinase II enzymatic activity has been detected in insulinoma cell lines and isolated pancreatic islets (Norling *et al.* 1994; Wenham *et al.* 1994). In an effort to distinguish which isoforms are expressed in insulin secreting cells, molecular techniques most recently have identified γ, δ, (δ_2, δ_6) (Mohlig *et al.* 1997; Breen *et al.* 1997) and a novel β_3 isoform (Urquidi *et al.* 1995). Moreover, glucose was demonstrated to activate CaM kinase II in isolated rat islets in a concentration-dependent manner that temporally correlated with the initial and sustained phases of insulin secretion (Fig. 5) (Wenham *et al.* 1994; Easom *et al.* 1997). Evidence for the suppression of glucose-induced insulin secretion by either a peptide inhibitor (Ammala *et al.* 1993) or pharmacological inhibitors (i.e. KN-62, KN-93) of CaM kinase II, have also implicated the enzyme in the regulation of insulin secretion (Wenham *et al.* 1992; Niki *et al.* 1993). However, complicating the interpretation of these data are reported nonspecific effects by these inhibitors which may interfere with Ca^{2+} channel activity (Clyne *et al.* 1995).

Investigation of the expression and subcellular localization of CaM kinase II is relevant to the possible role of CaM kinase II in insulin secretion and enable CaM kinase II, possessing such a broad substrate specificity, to obtain selectivity. In nerve terminals, a synaptic vesicle-associated form of CaM kinase II binds the COOH-terminal region of synapsin I and while catalytically indistinguishable from the soluble form, exhibits distinct characteristics in its association with the vesicle membrane and with synapsin I (Benfenati *et al.* 1996). In brain synaptosomes, CaM kinase II was predominantly associated with the cytoskeleton (Sahyoun *et al.* 1985) and upon microtubule purification under conditions that preserve kinase activity, CaM kinase II was determined to be endogenous to microtubules (Vallano *et al.* 1985). In rat forebrain extracts, activated CaM kinase II induced enhanced binding to forebrain proteins of 190 kDa and 140 kDa which were localized to a crude particulate/cytoskeletal fraction (McNeill *et al.* 1995). CaM kinase II association with the cytoskeleton has also been witnessed in cultured β-cell lines such as RINm5F and INS-1

FIG. 5. **Correlation of the activation of CaM kinase II by glucose with the initiation of insulin secretion in perifused rat islets.** Panels A and B: Islets (150-200/chamber) were pre-perifused with basal KRB containing 3 mM glucose for 30 min. At the indicated arrow, islets were perifused with the same medium (○) or KRB containing 17 mM glucose (●). Perifusates (1-5ml) were collected for the determination of insulin content (panel A). At the indicated times, islets were recovered from selected chambers, homogenized and assayed for CaM kinase II activation (panel B). The activity of CaM kinase II in the absence of Ca^{2+}/calmodulin (autonomous activity) expressed as a percentage of total activity in the presence of Ca^{2+} was used as a measure of enzyme activation. Panel C: Panel A and panel B have been superimposed.


A
B
C
Insulin Released (μ U/islet)
CaM Kinase II Activation
(% autonomy)
17 mM Glc
3 mM Glc
CaM Kinase II
Insulin Secretion
Time (min)

(Mohlig *et al.* 1997) and is in agreement with data on the distribution of CaM kinase II activity (Landt *et al.* 1982; Ashcroft *et al.* 1990; Norling *et al.* 1994). Furthermore, more detailed analyses of the subcellular localization of CaM kinase II δ isoform in INS-1 cells showed comigration of the kinase with insulin granules, suggestive of a possible association (Mohlig *et al.* 1997). In agreement with these data, recent findings from this laboratory have also indicated the presence and activity of CaM kinase II in preparations of purified secretory granules from insulinoma tissue (Easom *et al.* 1998). Finally, microscopic techniques that enabled visualization of secretory granule movement in hamster pancreatic β-cells (HIT T15), resulted in the proposal that protein phosphorylation by Ca^{2+}/calmodulin-dependent kinases played a positive role in the control of insulin granule movements (Hisatomi *et al.* 1996).

Hypothesis --- While increasing evidence supports an involvement of CaM kinase II in insulin secretion, a thorough comprehension of the physiological role of CaM kinase II in the mediation of this process can only be attained subsequent to the identification of its endogenous targets and their functional alteration upon phosphorylation. Accordingly, the development of this dissertation centered on the hypothesis that two prominent neuronal substrates of CaM kinase II that might also best influence secretory events, microtubule-associated protein 2 (MAP-2) and synapsin I, serve as endogenous substrates for CaM kinase II in the β-cell. Moreover, pertinent to this dissertation, is that the functional regulation of MAP-2 and synapsin I be dependent on the phosphorylation of each, specifically by CaM kinase II. Though there is a strong correlation between CaM kinase II activation and the subsequent secretion of insulin, the mechanisms by which CaM kinase II effects hormonal secretion remain largely undefined. The interaction between CaM kinase II and its endogenous substrates, MAP-2 and synapsin I, most likely effecting cytoskeletal organization and dynamics, may be an important intermediate step in the secretion process.

Research of this type contribute and aid in the comprehension of the regulatory role of CaM kinase II in insulin secretion, potentially improving the understanding of the mechanisms involved in insulin secretion and ultimately of the disorder, diabetes.

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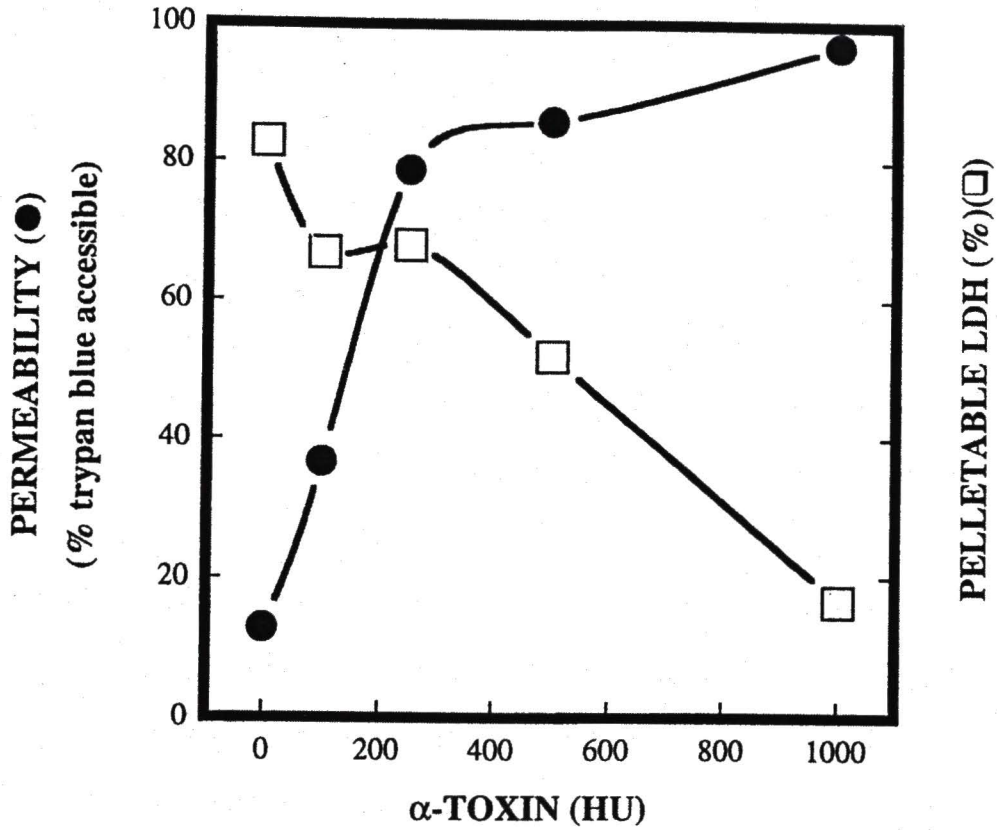
CHAPTER II

EXPERIMENTAL MODEL DEVELOPMENT

Permeabilization --- Classically, experiments designed to study *in situ* phosphorylation involve labeling the cells by incubation with large amounts of $^{32}P_i$ for long periods (3 - 4 hr). This was undesirable for the present system for a number of reasons. The proteins to be examined are able to serve as substrates for many kinases. Consequently, background or basal phosphorylation by other kinases would impair detection of small changes in phosphorylation. Specifically, MAP-2 exhibits a high basal amount of phosphate, such that changes in the total amount of phosphate incorporated in the immunoprecipitated MAP-2 would not be detectable if the entire endogenous ATP pool were radiolabeled. Also, still questionable, is the effect of glucose on the specific activity of ATP. Thus, to circumvent these potential problems, βTC3 cells were permeabilized prior to experimental treatment to permit access of high specific activity ATP. The permeabilization of βTC3 by *Staphylococcus aureus* toxin, α-hemolysin, induces the formation in the plasma membrane of pores of defined diameter (~ 2 nm) permitting ions and nucleotide access to the intracellular space without the loss of intracellular proteins. The initial amount and time of incubation with α-toxin was determined by assay of retention of the cytosolic enzyme marker, lactate dehydrogenase (LDH) versus trypan blue permeability (Fig. 1). Efficiency of permeabilization was monitored by visualizing trypan blue permeabilization to $\geq$ 60%. Incubation at 37 °C for 15 min with a concentration of 125 - 200 U/10^6 cell/0.1 ml Ca^{2+}-free permeabilization buffer of α-toxin was determined optimal with regard to permeabilization and loss of LDH from βTC3 cells.

FIG. 1. **Determination of optimal conditions for permeabilization of βTC3 cells by α-toxin.** The optimal number of hemolytic units (HU) was determined by plotting the visualized percentage of trypan blue permeability versus assayed pelletable lactate dehydrogenase in βTC3 cells.


PERMEABILITY (●)
(% trypan blue accessible)
PELLETABLE LDH (%)(□)
α-TOXIN (HU)
0
20
40
60
80
100
0
200
400
600
800
1000

Immunoprecipitation --- Once the initial phase of this involved protocol, permeabilization of βTC3 cells, was complete, the cells would be treated according to experimental conditions in the presence of [γ-^{32}P]ATP. The protein of interest would be isolated by immunoprecipitation, subjected to SDS-polyacrylamide electrophoresis, and ultimately analyzed by two-dimensional tryptic phosphopeptide mapping (Fig. 2). Unique to the current studies, the burgeoning protocol required an evaluation and optimization at each phase in order to achieve a successful conclusion.

Anti-MAP-2 and anti-synapsin I antibodies were generously provided by Dr. Michael Landt (Washington University School of Medicine, St. Louis, MO). Immunoblots were performed (ECL by Amersham) characterizing these antibodies in βTC3, an established cultured β-cell tumor line, and in isolated rat pancreatic islets, relative to the positive controls of brain homogenates. Determining the optimum dilution of each antibody for immunoblots similarly aided in determining the antibody ratio for effective immunoprecipitations.

Additional factors were considered in regard to the quality of the immunoprecipitations. To evaluate the specificity of the antibodies and the efficiency of the developing system, fractions were collected at various points throughout the immunoprecipitation procedure to be tested for immunoreactivity to the antibodies; specifically, after the lysis and centrifugation prior to adding the antibody (the homogenate), from the precleared supernatant after incubation with the antibody, and from the protein A-sedimented immunoprecipitate. Theoretically, the protein of interest would be present in the first fraction (prior to antibody) but absent from the precleared supernatant due to the capture of the antigen/antibody complex by protein A in the solid phase. As an additional check and positive control, immunoprecipitations were also conducted from brain simultaneously with the βTC3 cells. Moreover, routine silver staining of the gels

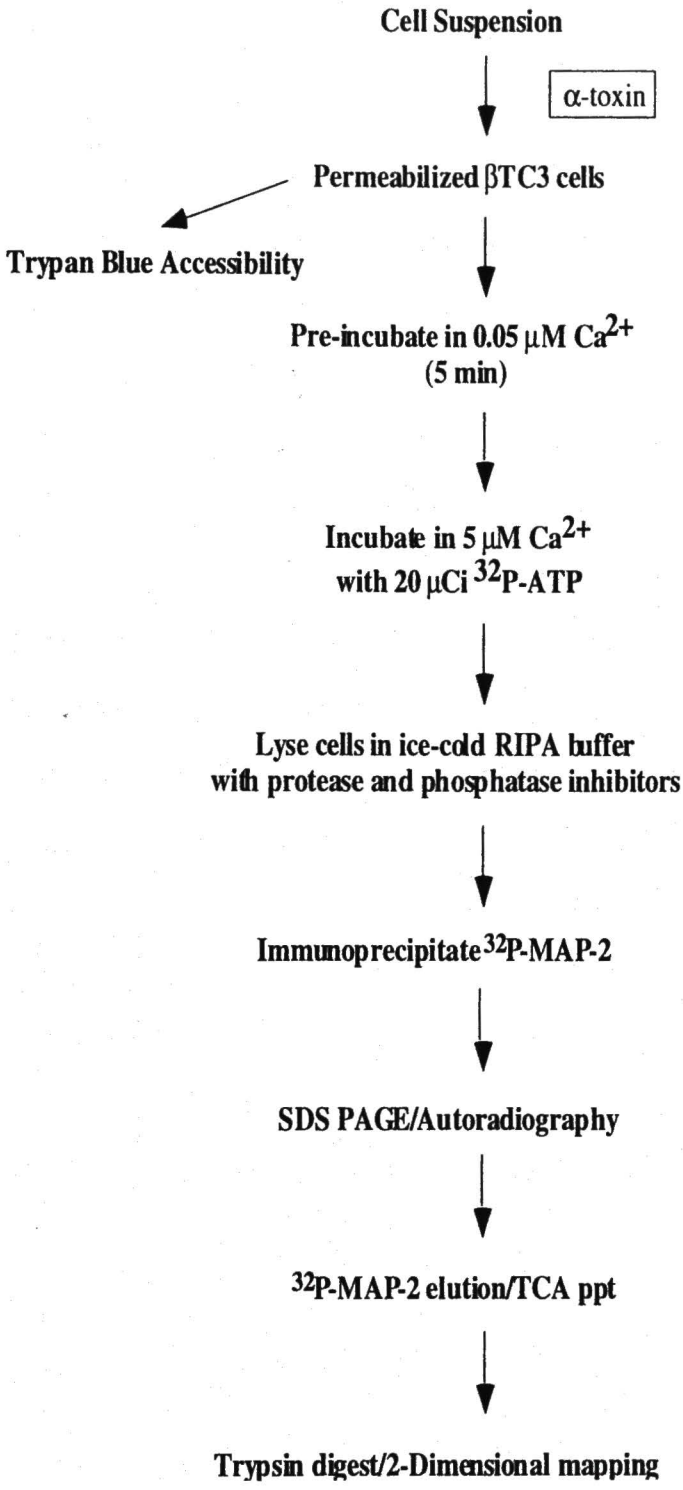
containing immunoprecipitates was performed to ensure standardized quantitation and loading of proteins.

Regarding other parts of the immunoprecipitation such as, centrifugation speed and time, and lysis buffer (RIPA) composition, slight modifications were made to tailor the procedure for these particular studies. For example, because these investigations dealt with phosphorylation events, lysing and washing buffers were supplemented with phosphatase and protease inhibitors i.e., sodium fluoride, sodium pyrophosphate, PMSF and leupeptin.

When developing the testing strategy for the different experimental treatments, the following factors were considered; preincubation time, termination efficiency, amount of specific activity (μCi), and ATP concentration of buffers. Concerned about the possible expiration of ATP during preincubation and the specific activity of [γ-^{32}P]ATP, the preincubation time was reduced and immunoprecipitations evaluated examining varying ATP concentrations. Additionally, because of the temporal nature of phosphorylation events, the physical manipulation of the samples between steps necessitated rapid and efficient handling. Ultimately, immunoprecipitation conditions were optimized for specific activity of [γ-^{32}P]ATP, cell number, and antigen/antibody/protein A ratios.

The immunoprecipitated ^{32}P-labeled MAP-2 or synapsin I could then be eluted from the gels, digested by trypsin and subjected to two dimensional phosphopeptide mapping for analysis of specific phosphorylated sites. These processes too required optimization and consideration at various steps. The most apparent elements of this last phase of the protocol for producing significant differences in results were the conditions for the electrophoresis and chromatography (i.e.; buffers, incubation times, and voltage).

FIG. 2. **Experimental protocol for the determination of calcium-induced phosphorylation of MAP-2 in α-toxin permeabilized βTC3 cells.**


Cell Suspension
α-toxin
Permeabilized βTC3 cells
Trypan Blue Accessibility
Pre-incubate in 0.05 μM Ca^{2+}
(5 min)
Incubate in 5 μM Ca^{2+}
with 20 μCi ^{32}P-ATP
Lyse cells in ice-cold RIPA buffer
with protease and phosphatase inhibitors
Immunoprecipitate ^{32}P-MAP-2
SDS PAGE/Autoradiography
^{32}P-MAP-2 elution/TCA ppt
Trypsin digest/2-Dimensional mapping

CHAPTER III

CALCIUM-STIMULATED PHOSPHORYLATION OF MAP-2 IN PANCREATIC βTC3 CELLS IS MEDIATED BY Ca^{2+}/CALMODULIN-DEPENDENT KINASE II

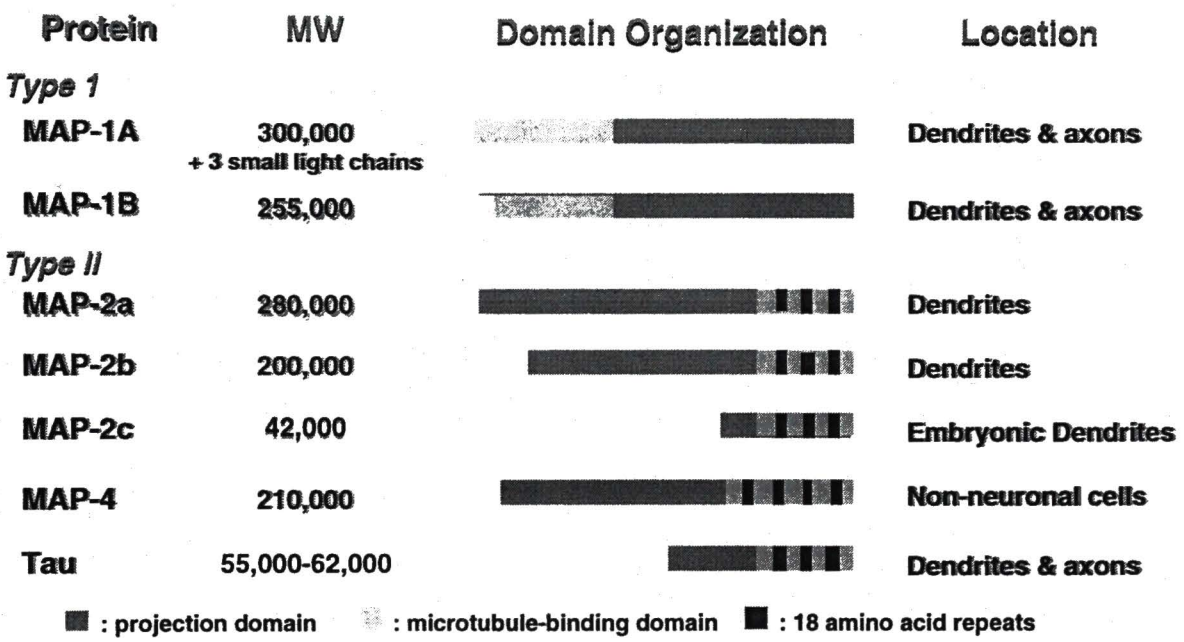
PREFACE

One of the best substrates of CaM kinase II *in vitro* that may also influence secretory events, is microtubule-associated protein-2, MAP-2. MAP-2 is a member of a larger family of microtubule-associated proteins that have been isolated based on their capacity to co-polymerize with microtubules during cycles of assembly and disassembly (Sloboda *et al.*1976). MAPs are asymmetric molecules that contain a globular cationic microtubule-binding domain and a longer projection domain which extends laterally from the microtubules and is thought to interact with other cytoskeletal components and cell membranes (Maccioni *et al.*1995). While these proteins share functional properties such as thermostability, ability to bind at the carboxy-terminal regulatory moiety on tubulin isoforms with a constant stoichiometry thereby stabilizing microtubules, and to interact with other cellular components, MAPs exhibit structural differences based on their amino acid sequence (Maccioni *et al.*1978). Accordingly, due to distinct microtubule-binding domains, MAPs have been divided into two groups. The first group is made up of: MAP-2, which consists of the high molecular weight doublet, 2A and 2B, of approximate molecular masses of 270 kDa, and 2C, a smaller 70 kDa protein; tau, of intermediate size (about 55-62 kDa); and MAP-4 (190-240 kDa). Members of this first group share common microtubule-binding regions which consist of 3 to 4 highly basic (pI = 10.5) 18-amino acid repetitive binding motifs near the carboxy-terminal domain of the molecule (Maccioni *et*

*al.*1995; Lewis *et al.*1988). The sole member of the second group, MAP-1 (1A, 1B and 1C each with an approximate molecular mass of 350 kDa) possesses a microtubule-binding domain that consists of 21 repeats more centrally located on the protein (Fig. 1). MAP-2 binds to microtubules, stimulating microtubule assembly and stabilizing them, once formed. It is proposed that besides stabilizing the cytoskeleton, MAPs may also function in regulating the associations of different components of the cellular network, spatially and temporally (Maccioni *et al.*1995). Supporting this proposal is the cumulative evidence that MAPs serve as intermediates between microtubules and other elements of the cytoskeleton as is demonstrated by the interaction of MAP-2 with actin filaments (Sattilaro, 1986). MAP-2 mediation of microtubules and interaction with intermediate filaments has also been examined by immunofluorescence (Clyne *et al.*1995). Due to the nature of MAPs association with tubulin, which polymerize to form microtubules, as well as other elements of the cell architecture, MAPs are considered regulatory proteins playing a major role in the modulation of the dynamic instability of microtubules and in associations with the cytoskeletal network and structured elements of the cell, such as organelles, centrosomes, nucleus, plasma membrane and secretory vesicles (Gelfand *et al.*1991; Maccioni *et al.*1995).

Functional regulation of MAP-2 occurs through phosphorylation. MAP-2 can be extensively phosphorylated, as witnessed by the presence of up to 46 phosphates mol/mol of MAP-2 (Tsuyama *et al.*1987) and serves as a substrate for a variety of kinases (Ainsztein *et al.*1994; Theurkauf *et al.*1982; Jefferson *et al.*1991). PKA directly associates with MAP-2 per its regulatory subunit and *in vitro* studies indicate that 10 - 13 phosphorylation sites on MAP-2 can be ascribed to PKA (Goldenring *et al.*1985). However, with an apparent K_m (0.15 μM) for CaM kinase II twentyfold less than that determined for PKA, MAP-2 is considered a better substrate for CaM kinase II with the stoichiometry of phosphorylation reported to be from 5 to over 20 mol of phosphate/mol of

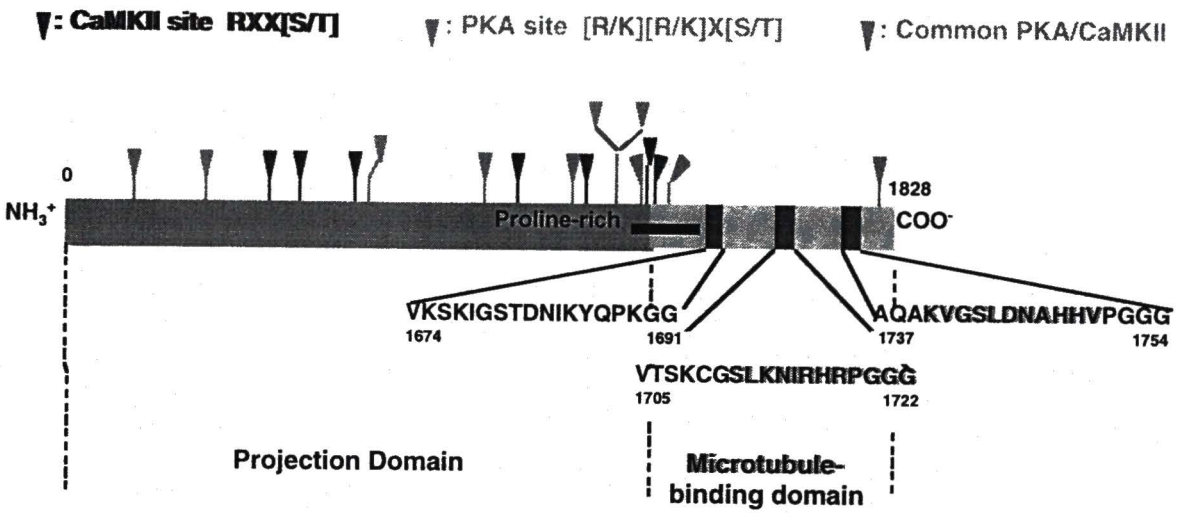
FIG. 1. **Domain structure of the family of microtubule-associated proteins.**


Protein
MW
Domain Organization
Location
Type I
MAP-1A
300,000
+ 3 small light chains
Dendrites & axons
MAP-1B
255,000
Dendrites & axons
Type II
MAP-2a
280,000
Dendrites
MAP-2b
200,000
Dendrites
MAP-2c
42,000
Embryonic Dendrites
MAP-4
210,000
Non-neuronal cells
Tau
55,000-62,000
Dendrites & axons
: projection domain
: microtubule-binding domain
: 18 amino acid repeats

MAP-2 (Fig. 2) (Yamamoto *et al.*1985). Additionally, sequencing of MAP-2 was accomplished during the late 1980s which revealed at least 13 possible phosphorylation sites for CaM kinase II (Kosik *et al.*1988). Phosphopeptide mapping has identified at least five sites phosphorylated by CaM kinase II *in vitro* (Goldenring *et al.*1985) and *in situ* in stimulated GH3 cells (Jefferson *et al.*1991). Phosphorylation of MAP-2 *in vitro* by CaM kinase II and PKA has been demonstrated to alter its function, such as promoting microtubule disassembly versus assembly, to reduce the rate and extent of microtubule assembly, and to reduce the interaction of microtubules with actin filaments (Brugg *et al.*1991; Sattilaro, 1986).

Speculation and some evidence target MAPs as an important intermediate link between associations with microtubules and secretory granules or other organelles. A study using centrifugation techniques to analyze the associations between microtubules and pituitary secretory granules, reported the preferential adsorption of MAPs to the secretory granules (Sherline *et al.*1977). In another study using dark-field light microscopy, secretory granules isolated from anglerfish endocrine pancreas only bound to microtubules assembled in the presence of MAPs and not to purified tubulin (Suprenant *et al.*1982). Numerous studies demonstrating that microtubule disrupting drugs inhibit secretion, also suggest the involvement of microtubules in the secretion process, possibly to mediate the intracellular movement of insulin-containing secretory granules (Santibanez *et al.*1995; Farshori *et al.*1997). Regulation of the ability of MAP-2 to bind to microtubules could alter the dynamic instability of microtubules. Phosphorylation appears to modulate the affinity of MAP-2 for microtubules (Brugg *et al.*1991; Sattilaro, 1986), but how this is achieved *in vivo* is still poorly understood. Because MAP-2 is a substrate for a variety of kinases and phosphatases, a coordinate sequence of selective phosphorylation of MAP-2 could contribute to the overall regulation of the cytoskeleton. MAP-2, in various phosphorylation states, was microinjected into rat fibroblasts which lack endogenous MAP-2 and bound to

FIG. 2. **Potential phosphorylation sites on MAP-2 for CaM kinase II and PKA based on known consensus sequences.**


: CaMKII site RXX[S/T]
: PKA site [R/K][R/K]X[S/T]
: Common PKA/CaMKII
0
1828
NH3+
Proline-rich
COO-
VKSKIGSTDNIKYQPKGG
1674
1691
AQAKVGSLDNAHHVPGGG
1737
1754
VTSKCGSLKNIRHRPGGG
1705
1722
Projection Domain
Microtubule-binding domain

endogenous microtubules only when it had been phosphorylated on a subset of its total phosphorylatable sites (Brugg *et al.*1991). It was suggested that the site of phosphate incorporation rather than the amount was the critical factor in determining the microtubule binding activity of MAP-2, and that the ability of MAP-2 to influence microtubule behavior may depend on the state of phosphorylation of MAP-2.

MAP-2 has been localized to dendrites, while tau, its closest relative, has been restricted to axons. MAP-2 and tau exhibit 67% amino acid similarity within their carboxy-terminal microtubule binding domain (Lewis *et al.*1988). Interestingly, both tau and MAP-2 are able to induce neurite-like processes in normally round cells without extensions (Thaler *et al.*1996). Considerable work has been conducted on tau and the characteristics of its interaction with microtubules (Gustke *et al.*1994). Phosphorylation at specific sites located within the microtubule-binding region of tau has been demonstrated to reduce the binding of tau to microtubules (Biernat *et al.*1993). Phosphorylation by CaM kinase II has been identified in the microtubule-binding region of tau and in the C-terminal tail of the protein outside of the region of internal microtubule-binding repeats (Litersky *et al.*1996). The phosphorylation by CaM kinase II of Ser ^{405} in the C-terminal area of tau versus the other potential sites available to three separate kinases (PKA, PKC, CKII), structurally modified tau making the protein long and stiff (Lichtenberg *et al.*1988). It was suggested that this induced conformational change may influence the binding of tau to microtubules, possibly reducing its affinity for microtubules (Steiner *et al.*1990). Because sequencing revealed that MAP-2 and tau have considerable homology, specifically that they share several repeated regions in tandem corresponding to their microtubule-binding domain (Lewis *et al.*1988), this together with the previous tau findings may prompt similar investigations for MAP-2. Accordingly, recent studies have attempted to determine the effect of kinase-mediated phosphorylation at specific sites in the microtubule-binding region of MAP-2, but this is only beginning(Ainsztein *et al.*1994).

In addition to possibly influencing secretory events by the mediation of the cytoskeletal architecture of the cell, MAP-2 may also affect organelle movement and transport by competing with microtubule motor molecules for microtubule binding. MAP-2, kinesin and cytoplasmic dynein have been demonstrated to bind to overlapping or closely adjacent sites on microtubules (Hagiwara *et al.* 1994). MAP-2 in competition with the motor molecules for binding to microtubules, displayed inhibition of dynein- or kinesin-driven microtubule gliding *in vitro* (Lopez *et al.* 1993).

Therefore, the cytoskeletal organization and the mechanisms that are involved in intracellular transport and secretion are most likely dependent upon regulatory proteins, such as MAP-2. This regulation in turn, is also apparently dependent on the degree of phosphorylation, and/or the site of phosphorylation by its respective specific kinases such as CaM kinase II. These qualities of MAP-2, together with the findings of the previous studies, prompted the ensuing investigation examining the potential of MAP-2 to act as an endogenous substrate for CaM kinase II in the pancreatic β-cell.

SUMMARY

An understanding of the role of CaM Kinase II in the pancreatic β-cell is dependent on the identification of its cellular targets. One of the best substrates of CaM Kinase II *in vitro* that could function in secretory events, is the microtubule-associated protein, MAP-2. By immunoblot analysis, a high molecular weight protein with electrophoretic properties characteristic of MAP-2, was identified in rat insulinoma βTC3 cells and isolated rat islets. In immunoprecipitation experiments employing α-toxin-permeabilized βTC3 cells, elevation of intracellular Ca^{2+} or addition forskolin, an adenylate cyclase activator, induced significant phosphorylation of MAP-2 *in situ*. The effect of Ca^{2+} was rapid, concentration-dependent and closely correlated with activation of CaM Kinase II under similar experimental conditions. H-89, a specific and potent inhibitor of cAMP-dependent protein kinase (PKA), prevented forskolin-induced MAP-2 phosphorylation but had little effect on MAP-2 phosphorylation stimulated by elevated Ca^{2+}. Phosphopeptide mapping revealed that the phosphorylation pattern observed *in situ* upon incubation of the βTC3 cells with increased free Ca^{2+}, was strikingly similar to that generated *in vitro* by CaM Kinase II, most notably with regard to the increased phosphate incorporated into one prominent site. These data provide evidence that MAP-2 is phosphorylated by CaM Kinase II in the pancreatic β-cell *in situ*, and that this event may provide an important link in the mediation of Ca^{2+}-dependent insulin secretion.

INTRODUCTION

Circumstantial evidence supports a functional role of the multifunctional, Ca^{2+}/calmodulin-dependent protein kinase II, CaM Kinase II, in the regulation of insulin secretion from the pancreatic β-cell. Principal within this evidence is the demonstration that glucose, the major physiological regulator of insulin secretion in rodents and humans (Hedeskov, 1980), activates CaM Kinase II in isolated rat islets in a concentration-dependent manner (Wenham *et al.* 1994) which temporally correlates with the initial and sustained phases of insulin secretion (Easom *et al.* 1997). Other data utilizing pharmacological inhibitors (*i.e.* KN-62, KN-93) of this enzyme have also implicated CaM Kinase II in the regulation of insulin secretion (Wenham *et al.* 1992; Niki *et al.* 1993) although conclusions made from such studies are complicated by demonstrated non-specific effects of these drugs (Clyne *et al.* 1995; Li *et al.* 1992). Another study that reports the inability of KN-62 to inhibit Ca^{2+}-induced insulin secretion from the permeabilized β-cell (Li *et al.* 1992) argues, however, against a role of CaM Kinase II in the insulin secretory process.

Irrespective of the relevance of CaM Kinase II to the β-cell secretory process, the understanding of the physiological consequence of the activation of CaM Kinase II is dependent on the identification of target substrates in the β-cell. A large number of cellular proteins are phosphorylated by CaM Kinase II *in vitro* (Braun *et al.* 1995) but of these, relatively few have been proven as legitimate substrates *in situ*. Prominent among this latter group, however, is the microtubule-associated protein-2 (MAP-2) which has been shown to be phosphorylated by CaM Kinase II in GH3 cells (Jefferson *et al.* 1991) or hippocampal slices (Diaz-Nido *et al.* 1993) stimulated with depolarizing concentrations of potassium. MAP-2 is a member of a larger family of microtubule-associated proteins that have the capacity to regulate reversible polymerization and stability of microtubules through

their affinity for tubulin (Sloboda *et al.*1976) as well as their interaction with other cellular structures such as actin (Sattilaro, 1986). This regulatory capacity is in turn controlled by the phosphorylation state of MAP-2, at least *in vitro* (Raffaelli *et al.*1992). Although a minimal extent of MAP-2 phosphorylation appears to be essential for MAP-2 function (Brugg *et al.*1991), phosphorylation by specific kinases *in vitro* has resulted in reduced affinity to microtubules, reduced rate and extent of assembly, accentuated disassembly, and reduced interaction of microtubules with actin filaments (Maccioni *et al.*1995). In optimal conditions, isolated MAP-2 has been demonstrated to incorporate phosphate to the level of 46 mol/mol of MAP-2 (Tsuyama *et al.*1987). Although MAP-2 is phosphorylated by multiple protein kinases including the phospholipid-dependent protein kinase C (PKC) (Ainsztein *et al.*1994) and the cAMP-dependent protein kinase (PKA) (Theurkauf *et al.*1982), MAP-2 is considered one of the best substrates for CaM Kinase II with the stoichiometry of phosphorylation reported to be from 5 to over 20 mol of phosphate/mol of MAP-2 (Yamamoto *et al.*1985).

Based on the established involvement of the microtubule network in insulin secretion (Somers *et al.*1979; Lacy *et al.*1972; Lacy *et al.*1975; Pipeleers *et al.*1976), and the suspected association of CaM Kinase II with the cytoskeleton of the β-cell (Harrison *et al.*1982), it was of interest to evaluate the potential of this enzyme to phosphorylate MAP-2 in these cells. Preliminary studies have established that CaM Kinase II can be efficiently activated by Ca^{2+} in the permeabilized β-cell. Therefore, to counter the inherent problem of a high level of basal MAP-2 phosphorylation, this model has been chosen to permit the study of phosphate incorporation from a high specific activity radionucleotide pool on a 'silent' background. The correlation of MAP-2 phosphorylation to CaM Kinase II activation, and CaM Kinase II activation to glucose-induced secretion, supports the hypothesis that a calcium-induced phosphorylation of MAP-2 by CaM Kinase II may function as an important intermediate step in insulin secretion.

EXPERIMENTAL PROCEDURES

Materials --- βTC3 cells were obtained from Dr. Shimon Efrat (Albert Einstein College of Medicine, New York). RPMI-1640, glutamine, antibiotics, trypsin/EDTA and fetal bovine serum were purchased from Life Technologies, Inc. (Gaithersburg, MD). Protein A-Sepharose, monoclonal anti-MAP-2 (clone HM-2), pure MAP-2, and α-hemolysin (*Staphylococcus aureus* α-toxin) were purchased from Sigma Chemical Co. (St. Louis, MO). From Worthington Biochemical Corp. (Freehold, NJ), ribonuclease A and TPCK-treated trypsin were acquired. K252a was purchased from LC Laboratories (Woburn, MA); H-89 and KN-93 were obtained from Calbiochem (La Jolla, CA). Forskolin was purchased from Research Biochemicals International (Natick, MA). [γ-^{32}P]ATP was purchased from DuPont NEN (Boston, MA). Autocamtide-2, sequence KKALRRQETVDAL (Hanson *et al.*1989) was synthesized by Bio-Synthesis, Inc. (Lewisville, TX). Anti-MAP-2 polyclonal antibody was raised against a heat-stable preparation of rat brain MAP-2 prepared by the method of Fellous *et al.*(Fellous *et al.*1977); the resulting anti-sera were purified to an IgG fraction enriched in anti-MAP-2 by chromatography on MAP-2-agarose. Mouse recombinant Ca^{2+}/calmodulin protein kinase IIα was generously provided by Dr. Roger Colbran (Vanderbilt University Medical Center, Nashville, TN). cAMP-dependent protein kinase catalytic subunit from bovine heart was donated by Dr. Ben Harris (UNTHSC, Fort Worth, TX). All other chemicals were of the finest reagent grade available.

Cell Culture and Permeabilization --- βTC3 cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 μg/ml penicillin, and 50 μg/ml streptomycin at 37 °C under an atmosphere of 5% CO_2. In preparation for permeabilization, βTC3 cells were detached (Trypsin/EDTA) and equilibrated in suspension

in culture medium for a minimum of 2 h. Following a brief centrifugation, the cells were washed twice with Ca^{2+}-free Krebs-Ringer Bicarbonate/Hepes buffer (25 mM Hepes pH 7.4, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl and 1 mM $MgCl_2$) containing 1 mM EGTA, 6 mM Glucose and 0.1% bovine serum albumin. After counting, permeabilization was initiated by the addition of *Staphylococcus aureus* toxin, α-hemolysin, to a concentration of 125-200 U/10^6 cell/0.1 ml Ca^{2+}-free permeabilization buffer (20 mM Hepes, pH 7.0, 140 mM potassium glutamate, 5 mM NaCl, 4 mM $MgSO_4$, 1 mM EGTA and 300 μM Na_2ATP). Permeabilization was conducted at 37 °C for 15 min with the efficiency monitored by visualizing trypan blue accessibility to >60%, and then terminated by the addition of ice-cold Ca^{2+}-free permeabilization buffer (washing twice). Cells were resuspended in permeabilization buffer containing 0.05 μM Ca^{2+} and placed on ice prior to experimental treatments. Free Ca^{2+} concentrations in incubation buffers were determined using a Ca^{2+}-electrode (Orion) calibrated against known standards as described by Bers (Bers, 1982). The permeabilization of βTC3 by α-toxin induces the formation in the plasma membrane of pores of defined diameter (~ 2 nm) permitting ions and nucleotide access to the intracellular space without the loss of intracellular proteins (Ahnert-Hilger, 1994).

Isolation of Pancreatic Islets --- Pancreatic islets were isolated from male Wistar rats (Harlan Sprague Dawley, (Indianapolis, IN) by collagenase P (Boehringer Mannheim, Indianapolis, IN) digestion and subsequent enrichment by centrifugation on a Ficoll gradient as described previously (Wenham *et al.*1994).

Immunoblot Analysis --- Immunoblot analyses were performed on nitrocellulose membranes using a Western-Light™ Protein Detection Kit (Tropix; Bedford, MA). Incubations with primary antibodies (rabbit polyclonal or monoclonal anti-MAP-2) were conducted overnight at 4 °C in blocking buffer.

Assay of CaM Kinase II Activity --- For the determination of CaM Kinase II activation, 5 x 10^5 permeabilized cells were incubated in buffer (500 µl) containing varying concentrations of free Ca^{2+} for 1 min at 37 °C. CaM Kinase II activity was assayed in sonicated homogenates using autocamtide-2 as substrate, by a method described previously (Babb *et al.*1996). $^{32}P_i$ incorporation into autocamtide-2 was determined by Cerenkov radiation (Beckman). The activity of CaM Kinase II in the absence of Ca^{2+}/calmodulin (autonomous activity) expressed as percentage of total activity in the presence of Ca^{2+} was used as a measure of enzyme activation.

MAP-2 Phosphorylation and Immunoprecipitation In situ --- Immunoprecipitation conditions were optimized for specific activity of [γ-^{32}P]ATP, cell number, MAP-2 antibody/protein A ratio and degree of permeabilization. Permeabilized βTC3 cells (approx. 2 x 10^6 per condition) were preincubated at 37 °C for 5 min in 0.05 µM Ca^{2+} permeabilization buffer, including kinase inhibitors when appropriate. The cells were then pelleted and resuspended in 200 µl of either 0.05 µM or 5-10 µM Ca^{2+} permeabilization buffer with 300 µM [γ-^{32}P]ATP (specific activity, 0.333 Ci/mmole), containing kinase inhibitors or activators when appropriate, and incubated at 37 °C for the indicated times. Phosphorylation was terminated by brief centrifugation (8,000 xg), washing with ice-cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2) supplemented with phosphatase inhibitors (50 mM NaF, 10 mM sodium pyrophosphate), and finally resuspension of the cells in 300 µl of ice-cold RIPA buffer (0.01 M sodium phosphate, pH 7.2, 0.15 M NaCl, 1% NP-40, 1% Na deoxycholate, 0.1% SDS, 1 mM DTT) containing phosphatase and protease inhibitors (50 mM NaF, 10 mM sodium pyrophosphate, 1 mM PMSF, 50 µg/ml leupeptin). The cells were lysed in this RIPA buffer for 45 min at 4 °C on a rotating platform before clarification by

centrifugation (12 min at 100,000 xg at 4 °C). The supernatant was transferred to a clean tube and incubated with polyclonal anti-rat MAP-2 antibody (1:100 dilution) for 2 h at 4 °C. Preswelled and washed protein A-Sepharose was added (25 µl) and the incubation continued for another 2 h at 4 °C on the rotating platform. The immune complexes bound to protein A-Sepharose were pelleted by centrifugation (3 min at 8,000 xg at 4 °C) and the pellets washed twice with RIPA buffer. The immunoprecipitation pellets were resuspended in 35 µl of 2X SDS sample buffer (124 mM Tris-HCl, pH 6.7, 6 mM SDS, 4% 2-mercaptoethanol, 10% glycerol, 0.007% bromophenol blue), and boiled for 10 min. Dissociated protein A-Sepharose was removed by centrifugation and a portion (20 µl) of the supernatant was subjected to SDS polyacrylamide electrophoresis on a 5% gel. Selected gels were silver stained to verify equality of protein loading. Dried gels were developed by autoradiography and ^{32}P-incorporation into MAP-2 quantified by densitometry using Optimas 4.0 and Scanalytics, ZERODscan 1.0, video imaging software.

MAP-2 Phosphorylation In vitro --- Purified MAP-2 (20 µg) was phosphorylated by the cAMP kinase catalytic subunit, or mouse recombinant CaM Kinase IIα as described (Jefferson *et al.* 1991) with the following exceptions; the cAMP kinase mixture was without exogenously added $CaCl_2$, and the reaction volume of 50 µl contained [γ-^{32}P] ATP (2 Ci/mmol) and 500 ng of kinase. Reactions proceeded for 18 min at 30 °C and were terminated by rapid chilling on ice.

Two-dimensional Tryptic Phosphopeptide Mapping of MAP-2 --- For phosphopeptide mapping, ^{32}P-labeled MAP-2 was eluted from gel slices by incubation in 50 mM NH_4HCO_3, pH 7.3-7.6, initially supplemented with 1% β-mercaptoethanol and 0.1% SDS for 18 h at 25 °C, and then without supplement for a further 3 h. The eluates were pooled

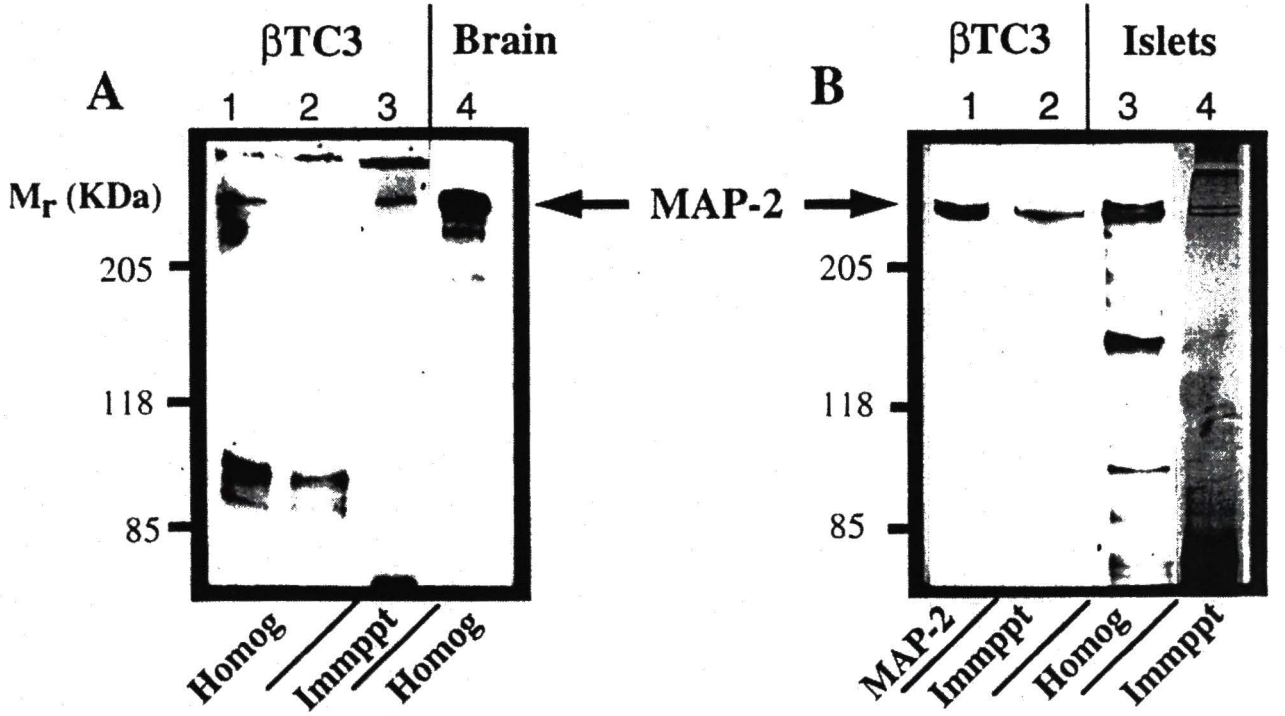
and the eluted MAP-2 precipitated by the addition of a final concentration of 16% TCA (1 h, on ice) in the presence of 20 μg heat-denatured RNAse as carrier. *In vitro* phosphorylated MAP-2 was similarly precipitated at this step. The precipitate was resuspended in oxidizing solution (50 μl of performic acid) and then digested by the addition of 10 μg TPCK-treated trypsin for 18 h at 37 °C and then another 10 μg for a further 2.5 h. After repeated lyophilizing, the proteolytic digests were resuspended in electrophoresis buffer (2.5% formic acid and 7.8% glacial acetic acid v/v) and spotted onto cellulose thin-layer plates. Two-dimensional separation of phosphopeptides by electrophoresis and chromatography was performed on a HTLE 7000 thin-layer electrophoresis apparatus (C.B.S. La Jolla, CA) as described (van der Geer *et al.*1994) except that the electrophoresis and chromatography steps were conducted at 1.3 kV for 25 min, and for 14 hr using a phosphochromatography buffer (37.5% n-butanol, 25% pyridine 7.5% glacial acetic acid v/v), respectively.

Statistical Analysis --- Data are expressed as the mean ± S.E. determined from at least 3 independent observations unless otherwise stated. Differences were assessed statistically through the employment of the most appropriate tests, either a two-way or one-way parametric ANOVA with Dunnetts multiple range test or with an independent t-test (SAS Institute, Cary, NC). $p<0.05$ indicates statistical significance.

RESULTS AND CONCLUSIONS

βTC3 Cells Express MAP-2 --- MAP-2 has been extensively characterized in mammalian brain where it is concentrated in dendritic processes (Olmstedt, 1986; De Camilli *et al.*1984; Caceres *et al.*1983) accounting for as much as 1% of the total cytoplasmic protein. In contrast, MAP-2 levels are much lower in non-neuronal tissues (Valdivia *et al.*1982) but demonstrated to be expressed in secretory cells, rat glioma (Koszka *et al.*1985), pituitary and PC12 (Valdivia *et al.*1982). By immunoblot analysis using a polyclonal anti-MAP-2 antibody, βTC3 cells were demonstrated to express a high molecular weight protein (M_r>205kDa) of electrophoretic mobility indistinguishable from MAP-2 purified from bovine brain (Fig. 3A, lane 1 vs. lane 4). This MAP-2-like protein was immunoprecipitated from βTC3 cell homogenates by this antibody as indicated by its disappearance from β-cell homogenates (Fig. 3A, lane 2) and its appearance in protein A-sedimented immunoprecipitates (Fig. 3A, lane 3). Immunoblot analysis of this immunoprecipitate using a monoclonal anti-MAP-2 antibody, confirmed the identity of this high molecular weight protein as MAP-2 (Fig. 3B, lane 2). That MAP-2 expression in βTC3 cells was not an artifact of β-cell transformation was supported by the presence of immunoreactive, immunoprecipitable MAP-2 in isolated rat islets (Fig.3B, lanes 3 & 4). It was noted however, that βTC3 cells express only a single form of MAP-2 in contrast to the characteristic doublet of MAP-2 (comprised of MAP-2A and 2B) observed in neurons (Schulman, 1984) and here demonstrated in islet immunoprecipitates (Fig. 3B, lane 4). These findings, coupled to the ensuing demonstration that this high molecular weight protein was capable of being phosphorylated by kinases known to phosphorylate MAP-2 *in vitro* (see below), established that βTC3 cells express MAP-2. Despite previous inferences to the presence of MAPs in the pancreatic β-cell (Pipeleers *et al.*1976), this study is believed to be the first demonstration that these cells express MAP-2. Closer scrutiny of

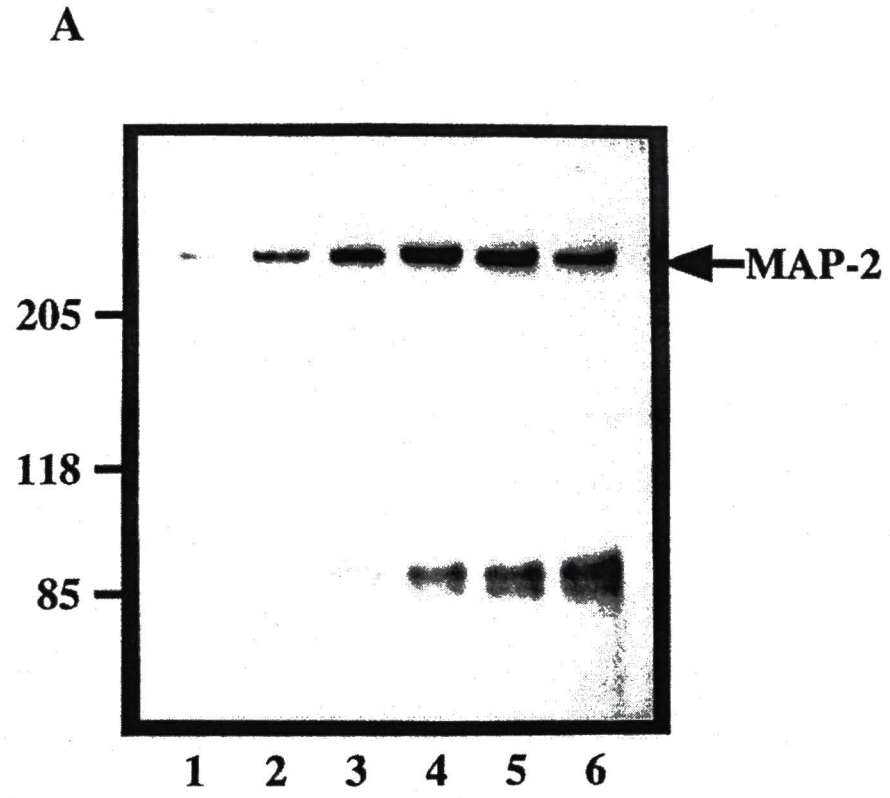
FIG. 3. **Expression of MAP-2 in βTC3 cells and isolated islets.** ***Panel A.*** Immunoblot analyses were performed using a rabbit anti-MAP-2 antibody at various stages of MAP-2 immunoprecipitation from βTC3 cells. Lanes 1 & 2: βTC3 homogenate (~60 μg protein) before and after addition of protein A-sepharose. Lane 3: immunoprecipitate (lower band represents dissociated antibody). Lane 4: whole rat brain homogenate (~14 μg protein). ***Panel B, Lanes 1 & 2:*** Immunoblot analysis was performed using a monoclonal anti-MAP-2 antibody. Lane 1: purified MAP-2 (0.1 mg). Lane 2: βTC3 cell immunoprecipitate from panel A. ***Panel B, Lanes 3 & 4.*** Isolated rat islets (50 per condition) were homogenized and subjected to immunoblot analysis (rabbit anti-MAP-2)(Lane 3) or immunoprecipitation followed by silver stain for protein content (Lane 4).

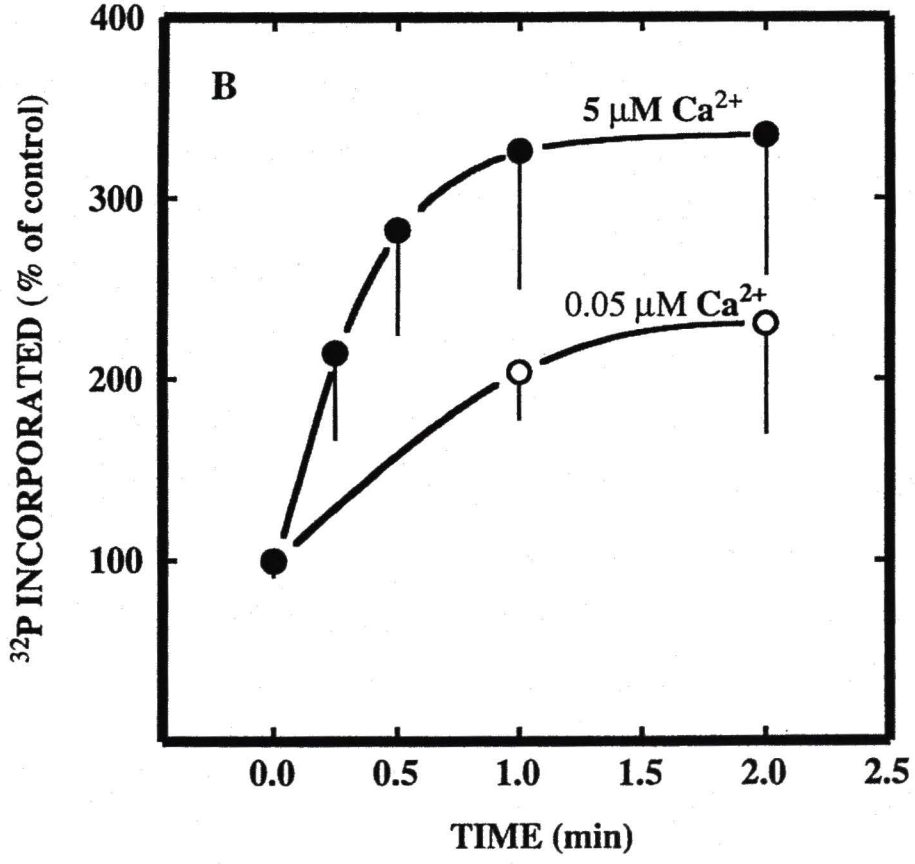

A
βTC3
Brain
1
2
3
4
M_r (KDa)
205
118
85
Homog
Immppt
Homog
MAP-2
B
βTC3
Islets
1
2
3
4
205
118
85
MAP-2
Immppt
Homog
Immppt

immunoblot analyses indicate that β-cells express MAP-2 to a lower extent (by a factor of 50-60) relative to whole brain extract and therefore similar to estimates from other non-neuronal tissues (Valdivia *et al.* 1982).

Time- and Calcium-dependent Phosphorylation of MAP-2 In situ --- The major objective of this study was to evaluate whether MAP-2 serves as a substrate for CaM Kinase II in the pancreatic β-cell. Sequence analysis of brain MAP-2 has identified 13 potential phosphorylation sites for CaM Kinase II based on the published consensus sequence RXX(S/T) (Kosik *et al.* 1988). At least 5 of these sites have been demonstrated to be phosphorylated by this kinase *in vitro* (Goldenring *et al.* 1985) and a similar number of sites observed in stimulated GH3 cells *in situ* (Jefferson *et al.* 1991). However, MAP-2 also serves as a prominent substrate for PKA (Theurkauf *et al.* 1983; Jefferson *et al.* 1991), and other known protein kinases (Tsuyama *et al.* 1986). Therefore, to circumvent anticipated difficulties in the detection of increased phosphate incorporation into MAP-2 as the result of the activation of selective protein kinases on a high background level of basal phosphorylation (Jefferson *et al.* 1991), βTC3 cells were permeabilized with α-toxin and radiolabeled [γ-^{32}P]ATP only introduced during incubation periods. This method of permeabilization was chosen to minimize the loss of intracellular proteins (Ahnert-Hilger, 1994). In the presence of 0.05 μM Ca^{2+} [to mimic the intracellular concentration of a resting β-cell (Gilon *et al.* 1995)] $^{32}P_i$ was incorporated into MAP-2 in a time-dependent manner (Fig. 4). This response likely reflected the activity of protein kinases involved in the maintenance of basal phosphorylation levels of MAP-2 which are thought to be required for the retention of protein function (Brugg *et al.* 1991). On elevation of the Ca^{2+} concentration to 5 μM (to promote the activation of CaM Kinase II) the extent of $^{32}P_i$ incorporation into MAP-2 was significantly increased; at the optimal time of 1 min, 5 μM

FIG. 4. **Calcium induced the time-dependent phosphorylation of MAP-2.** Permeabilized βTC3 cells were incubated in buffers containing free Ca^{2+} concentrations of 0.05 μM (○) or 5 μM (●) at 37 °C for the times indicated. MAP-2 was immunoprecipitated and phosphate incorporation was quantitated by autoradiography and densitometry. (A) Autoradiogram of immunoprecipitated MAP-2. Lane 1 is 0.05 μM Ca^{2+} for 0 min; lane 2, 3, 4, and 5 are 5 μM Ca^{2+} for 15 s, 30 s, 1 min and 2 min respectively; lane 6 is 0.05 μM Ca^{2+} for 2 min. The identity of the phosphoprotein (M_r ~ 89 kDa) co-immunoprecipitated with MAP-2 is not known. (B) Densitometric data is expressed as percentage of control (0.05 μM Ca^{2+} at 0 min). Each data point was determined from a minimum of 3 replicates; the majority of points were determined from 6 replicates. Ca^{2+} and time significantly affected the mean phosphorylation of MAP-2 relative to time zero, $p=0.04$ and $p=0.03$ respectively; however, the interaction of the two variables did not, $p=0.93$ (two-way parametric ANOVA Model I with replication).


A
MAP-2
205
118
85
1 2 3 4 5 6

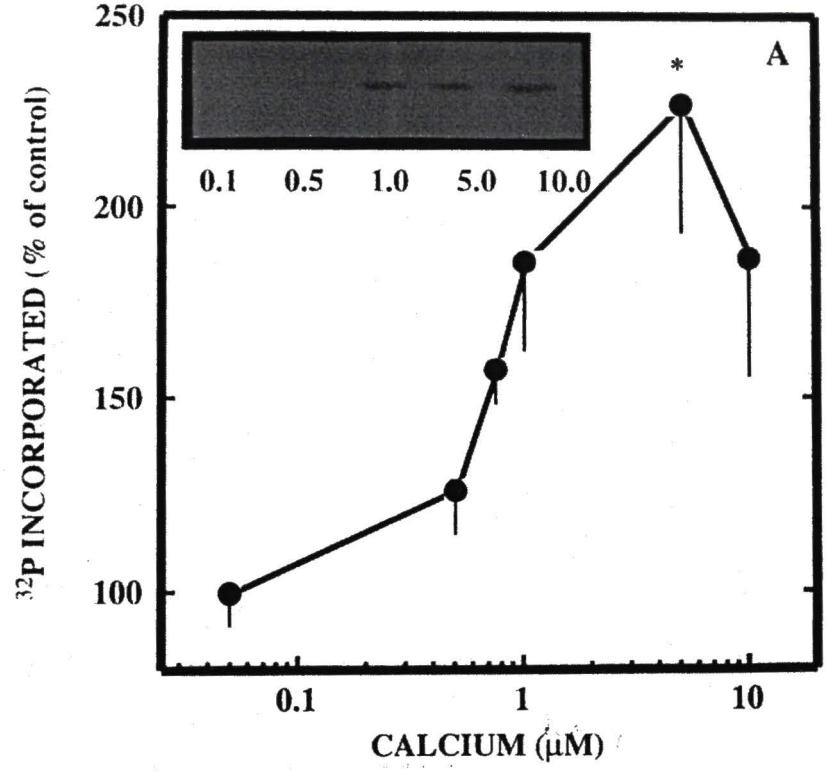

B
5 μM Ca2+
0.05 μM Ca2+
32P INCORPORATED (% of control)
400
300
200
100
0.0 0.5 1.0 1.5 2.0 2.5
TIME (min)

Ca^{2+} increased $^{32}P_i$ incorporation into MAP-2 by 326 ± 76% relative to time zero and by 163% relative to control cells incubated in the presence of 0.05 μM Ca^{2+}. An autoradiogram of immunoprecipitated MAP-2 under these experimental conditions is shown in Fig. 4A.

The phosphorylation of MAP-2 was also dependent on Ca^{2+} concentration. Thus, Ca^{2+} concentrations of 0.5 μM or greater were required to induce detectable MAP-2 phosphorylation (Fig. 5A) and half maximal phosphorylation was achieved at approximately 0.8 μM Ca^{2+}. As demonstrated in Fig. 5B, similar Ca^{2+} concentrations were required to activate CaM Kinase II under identical conditions. Again increases in free Ca^{2+} concentration beyond 0.5 μM were required to induce kinase activation and half maximal activation was achieved at approximately 1 μM Ca^{2+}, consistent with the known low affinity of this enzyme for Ca^{2+}/calmodulin relative to other Ca^{2+}-activated kinases (Hanson *et al.*1992). The similarity of these Ca^{2+} dependency profiles is consistent with a functional association of Ca^{2+}-dependent activation of CaM Kinase II with the phosphorylation of β-cell MAP-2 and is further substantiated by virtually identical Ca^{2+}-dependency of CaM kinase-mediated phosphorylation of brain MAP-2 conducted *in vitro* (Schulman, 1984).

The maintenance of a minimal level of cAMP is required to support glucose-induced insulin secretion from FACS-purified β-cells (Wang *et al.*1990; Schuit *et al.*1985) and other studies have localized an effect of cAMP to potentiate Ca^{2+}-induced insulin secretion to some distal step of the secretory process (Hughes *et al.*1987). Since MAP-2 may also serve as a substrate for PKA (Schulman, 1984) in the pancreatic β-cell, it was important to determine to what extent Ca^{2+}-induced phosphorylation of MAP-2 was contributed by the activation of this kinase. To this end, permeabilized cells were incubated in buffer containing 0.05 μM or 5 μM Ca^{2+} supplemented with forskolin (10 μM), a known activator of adenylate cyclase, and/or H-89 (5 μM), a specific inhibitor of PKA (Chijiwa *et al.*1990)(Fig. 6). In the presence of basal concentrations of Ca^{2+} (0.05 μM), forskolin

FIG. 5. **Calcium-dependent phosphorylation of MAP-2 (A) and activation of CaM Kinase II (B).** Permeabilized βTC3 cells were stimulated with increasing concentrations of calcium (0.05-10 μM) at 37 °C for 1 min. (A) Cells were then harvested and lysed for MAP-2 immunoprecipitation. The inset displays the autoradiogram of MAP-2 phosphorylation at the indicated Ca^{2+} concentrations (μM). Graphed is MAP-2 phosphate incorporation as determined by autoradiography and densitometry. The means of relative MAP-2 phosphorylation of the six calcium groups were significantly different (one-way parametric ANOVA, p=0.001), and the mean of the 5 μM Ca^{2+} group (*), as well as the 1 μM and 10 μM Ca^{2+} groups, were statistically distinct from the mean of the 0.05 μM Ca^{2+} group (Dunnetts, α=0.05). (B) Cells were homogenized for analysis of CaM Kinase II activation. CaM Kinase II activation was quantitated by the determination of the fraction of enzyme in the autophosphorylated, Ca^{2+}-independent form as previously described (Wenham *et al.* 1994). Autonomous CaM Kinase II activity was determined as described in *Materials and Methods*, and expressed as a percentage of Ca^{2+}-dependent CaM Kinase II activity.


A
250
200
150
100
*
0.1
0.5
1.0
5.0
10.0
^{32}P INCORPORATED (% of control)
0.1
1
10
CALCIUM (μM)

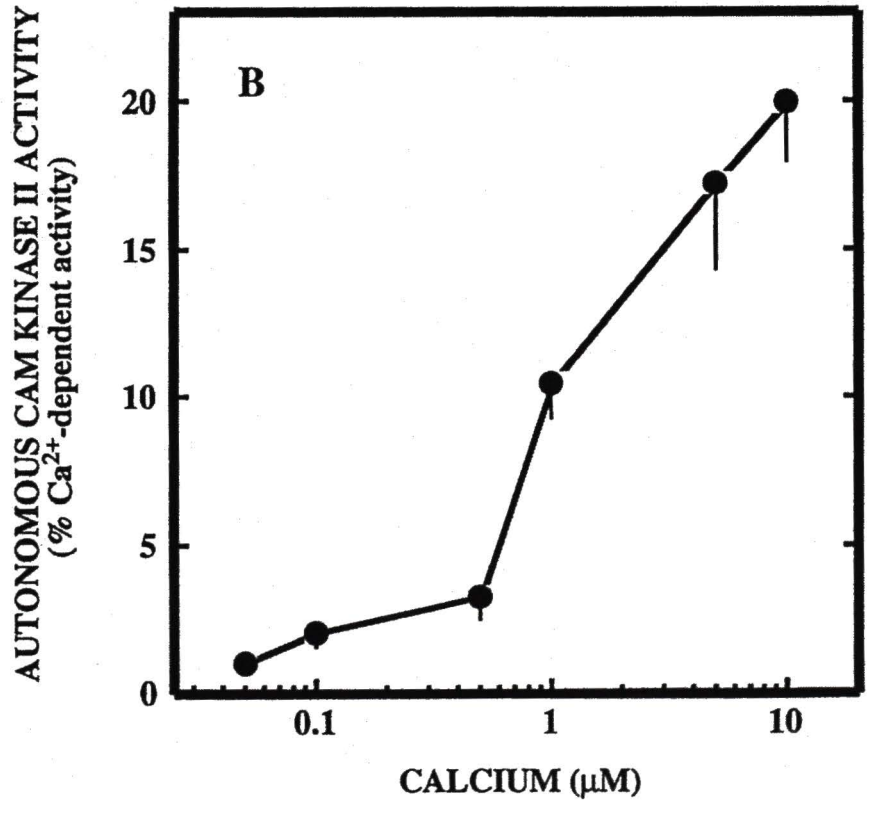
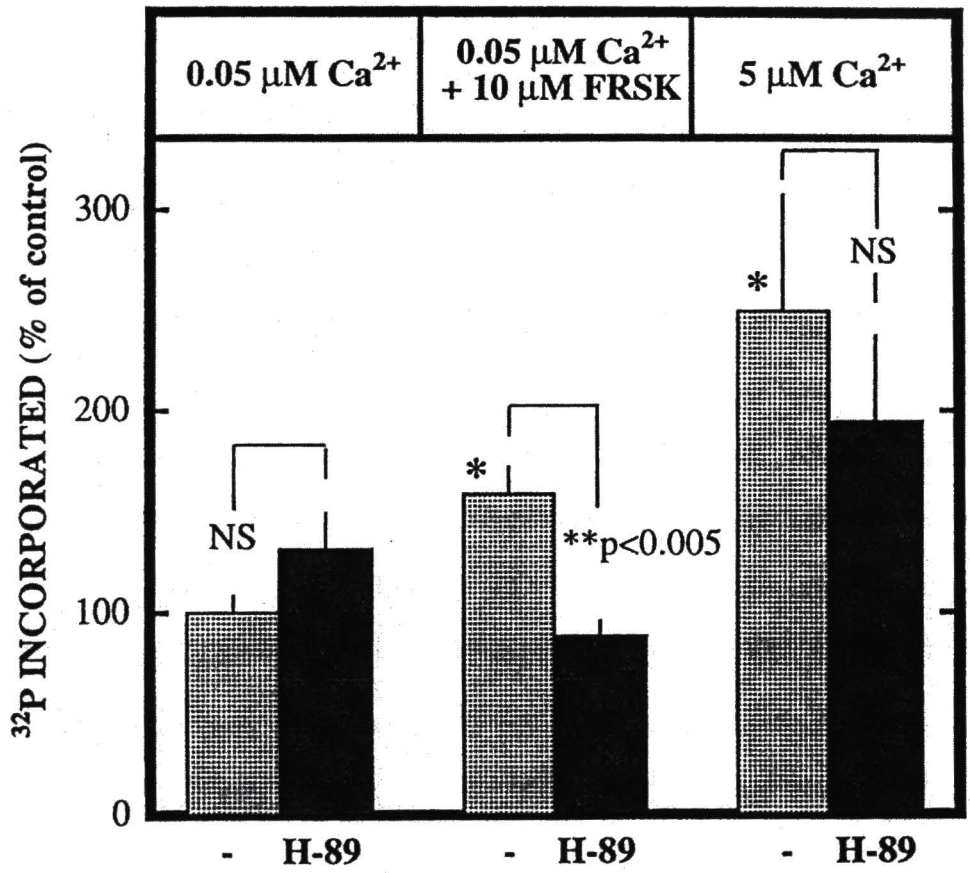

B
20
15
10
5
0
AUTONOMOUS CAM KINASE II ACTIVITY
(% Ca^{2+}-dependent activity)
0.1
1
10
CALCIUM (μM)

FIG. 6. **Calcium-induced phosphorylation of MAP-2 is independent of the activation of protein kinase A.** Permeabilized βTC3 cells were incubated with buffers containing free Ca^{2+} concentrations of 0.05 μM or 5 μM Ca^{2+}, supplemented with 10 μM forskolin and/or 5 μM H89. MAP-2 was then immunoprecipitated from cell lysates and phosphate incorporation determined by autoradiography and densitometry. * $p<0.02$ compared to control (0.05 μM Ca^{2+}), ** $p<0.005$ (independent t-test).


0.05 μM Ca2+
0.05 μM Ca2+ + 10 μM FRSK
5 μM Ca2+
32P INCORPORATED (% of control)
300
200
100
0
NS
*
**p<0.005
*
NS
- H-89
- H-89
- H-89

induced a significant phosphorylation of MAP-2 (160 ± 13% relative to control, p=0.004) which was totally abrogated by the inclusion of 5 μM H-89 (Fig. 6). As anticipated, forskolin had no significant effect on the activation state of CaM Kinase II in these cell preparations (data not shown). In contrast, MAP-2 phosphorylation induced by stimulatory concentrations of Ca^{2+} (5 μM), was only modestly (22%) reduced in the presence of H-89, an effect that was not statistically significant (p=0.48) (Fig. 6). Accordingly, H-89 (5 μM) had only modest effects on CaM Kinase II activity in βTC3 cell homogenates or on CaM Kinase II-mediated phoshorylation of purified MAP-2 *in vitro* (~15% inhibition in either case, date not shown). These observations demonstrate that the activation of PKA is capable of inducing MAP-2 phosphorylation in permeabilized βTC3 cells. This activation may contribute, although not significantly, to MAP-2 phosphorylation induced by 5 μM Ca^{2+}, a logical explanation being provided by the demonstrated presence, in the β-cell, of calmodulin-dependent adenylate cyclase and phosphodiesterase, activities that could bestow Ca^{2+}-dependent modulations of intracellular cAMP concentrations (Sharp, 1979).

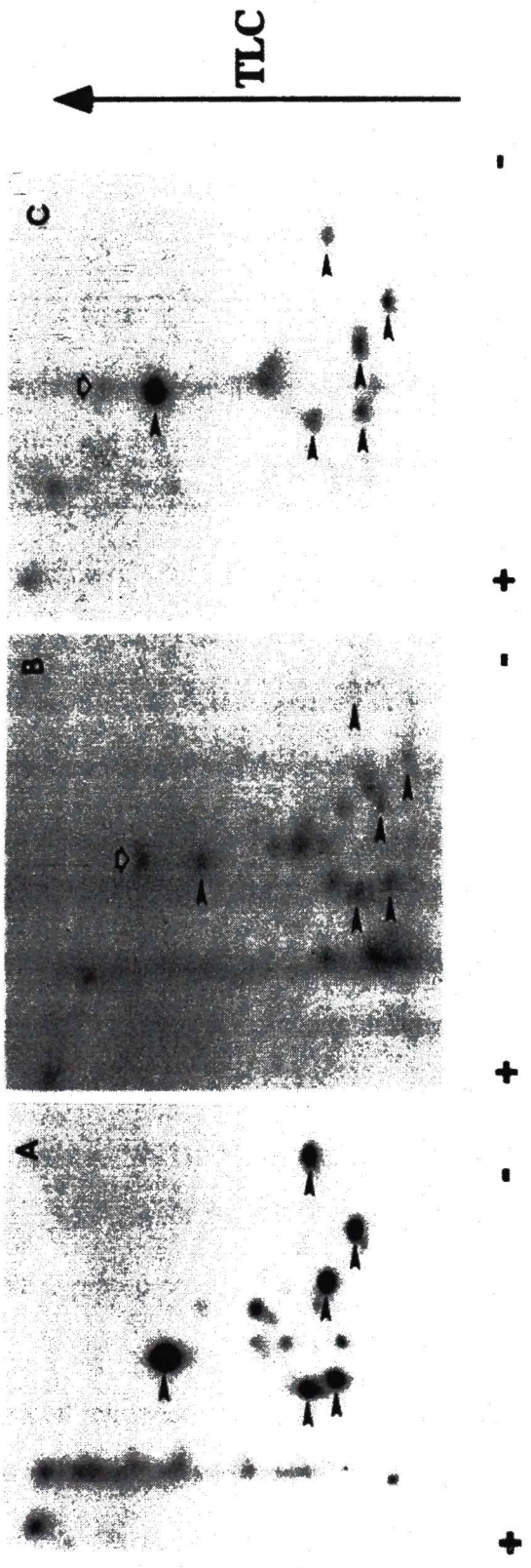
Identification of Site-Specific Phosphorylation of MAP-2 by 2-dimensional Phosphopeptide Mapping --- Attempts to support the hypothesis that Ca^{2+}-induced phosphorylation of MAP-2 was mediated by CaM Kinase II via the use of putative inhibitors of this enzyme, KN-93 and K252a, were thwarted by observed non-specific effects of these compounds. While KN-93 and K252a both abolished Ca^{2+}-induced phosphorylation of MAP-2, these compounds also significantly suppressed forskolin-induced phosphorylation of MAP-2 (data not shown). In light of the inability of forskolin to affect the activation state of CaM Kinase II, it was reasoned that these effects must reflect a lack of specificity of these compounds *in situ*. Therefore, in the absence of selective

inhibitors of CaM Kinase II, specific phosphorylation sites targeted in response to Ca^{2+} were determined by two-dimensional tryptic phosphopeptide analysis.

Through *in vitro* incubation with recombinant enzyme, 6 major and several minor phosphorylation sites for CaM Kinase II on purified brain MAP-2 were identified (Fig. 7A) consistent with previous reports (Jefferson *et al.*1991). Although initial studies were conducted using a neuronally expressed isoform of CaM Kinase II, *i.e.* CaM Kinase IIα, similar phosphopeptide patterns were generated from MAP-2 phosphorylated by a δ2 isoform, recently demonstrated to be prominently expressed in β-cells (Mohlig *et al.*1997). All of the major CaM Kinase II sites were evident in digests made from MAP-2 that had been immunoprecipitated from βTC3 cells stimulated in the presence of 5 μM Ca^{2+} (indicated by the arrowheads, Fig. 7B) as verified by comigration with *in vitro*-generated phosphopeptides (Fig. 7C). Not only do these data suggest that structural features of neuronal MAP-2 surrounding these phosphorylation sites are equivalent in the pancreatic β-cell protein but further imply that functional regulation of MAP-2 asserted by CaM Kinase II-specific phosphorylation may also be conserved.

Comparison of phosphopeptide digests generated from MAP-2 phosphorylated in the presence of basal Ca^{2+} concentrations (0.05 μM) or stimulatory Ca^{2+} concentration (5 μM) revealed significant differences. A representative experiment is illustrated in Fig. 8. Although some variation was observed between experiments, characteristic of most analyses was a marked (780±140% over control) Ca^{2+}-induced phosphorylation of a site central to the phosphopeptide map (indicated by a large hollow circle). Interestingly, this spot corresponded to the site most responsive to *in vitro* phosphorylation by purified CaM Kinase II (Fig. 7A) providing compelling evidence that MAP-2 serves as a substrate for this enzyme in βTC3 cells. In the indicated experiment, Ca^{2+} induced the net phosphorylation of other sites (labeled by a small "o") that corresponded to CaM Kinase II-specific sites (*cf.* Fig. 7A) but significant differences in phosphate incorporation into these

FIG. 7. **Two-dimensional tryptic phosphopeptide analyses of MAP-2 phosphorylation *in vitro* and *in situ*.** (A) Purified MAP-2 was phosphorylated by CaM Kinase IIα as described in *Materials and Methods*. (B) *In situ* phosphorylated MAP-2 was immunoprecipitated from permeabilized βTC3 cells stimulated for 1 min at 37 °C with buffers containing free Ca^{2+} concentrations of 5 μM. (C) Mix of *in vitro* and *in situ* phosphorylated MAP-2 (A and B, respectively). After tryptic digestion, the resultant peptides were separated by electrophoresis in the horizontal dimension and ascending chromatography in the vertical dimension. Cerenkov counts/min loaded onto thin-layer plates were 1000 cpm (A), 300 cpm (B), and 300 cpm each (C). *Arrowheads* indicate major phosphopeptides observed in MAP-2 phosphorylated by CaM Kinase IIα *in vitro* (A), and also seen in Ca^{2+}-induced *in situ* phosphorylation of MAP-2 (B), as well as in the mix (C). The *open arrowhead* indicates a phosphopeptide that, while present upon *in situ* stimulation, is not phosphorylated by CaM Kinase IIα *in vitro*.


TLC
C
B
A
-
+
-
+
-
+

sites was not uniformly observed in all experiments. It is possible that these additional sites are not as readily available to the enzyme *in situ* relative to *in vitro* conditions and suggest that they are secondary to the site described above. These data therefore demonstrate that CaM Kinase II phosphorylates at least one site on MAP-2 establishing this protein as a substrate for this enzyme in the β-cell.

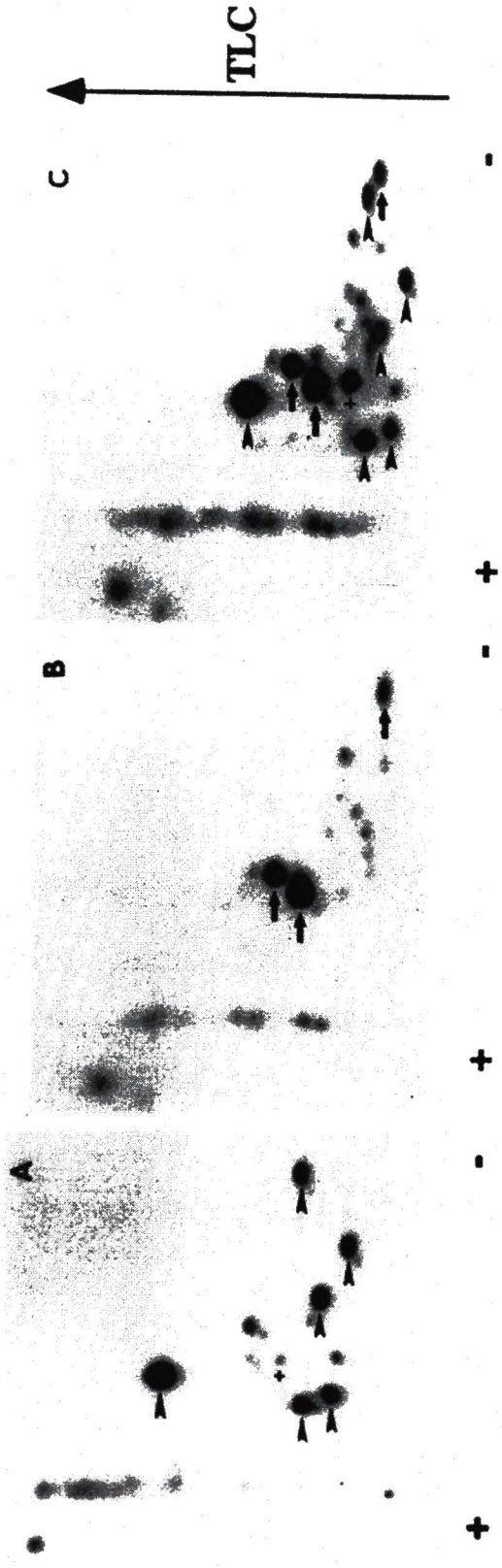
Ca^{2+} induced several changes in the phosphorylation of MAP-2 that cannot be ascribed to CaM Kinase II. One such change was characterized by a net dephosphorylation (Fig. 8, cross symbol) implicating the action of a Ca^{2+}-dependent phosphatase, *e.g.* calcineurin, as has been previously reported (Goto *et al.*1985; Ferreira *et al.*1993). Ca^{2+} also induced the phosphorylation of sites of similar migration to major sites targeted by PKA *in vitro* (Fig. 8, indicated by "p") that were clearly distinct from sites targeted by CaM Kinase II (Fig. 9). This suggests that these may represent cAMP-induced phosphorylation events consistent with the ability of H-89 to modestly inhibit MAP-2 phosphorylation. Alternatively, they could represent sites phosphorylated by other Ca^{2+}-sensitive protein kinases such as PKC (Easom *et al.*1990) or MAP kinase (Frodin *et al.*1995). To what extent the function of MAP-2 is dependent on phosphorylation at multiple sites targeted by distinct kinases is not clear although it is likely that the site of phosphate incorporation rather than the overall amount is the critical factor for the specific regulation of MAP-2 (Brugg *et al.*1991). Nevertheless, because of its ability to act as a common substrate for both CaM Kinase II and PKA, as well as other kinases/phosphatases, MAP-2 may provide a point of signal convergence for the integrated control of insulin secretion.

A considerable body of evidence generated from the use of microtubule disrupting drugs support a role for the dynamic assembly/disassembly of microtubules in the mechanism of insulin secretion (Farshori *et al.*1997; Lacy *et al.*1975; Somers *et al.*1979;

FIG. 8. **Two-dimensional tryptic phosphopeptide analyses of basal and stimulated *in situ* phosphorylated MAP-2.** *In situ* phosphorylated MAP-2 was immunoprecipitated from permeabilized βTC3 cells incubated with buffers containing free Ca^{2+} concentrations of 0.05 μM (A) and 5 μM (B) and subjected to tryptic digestion subsequent to two-dimensional phosphopeptide mapping as described in *Materials and Methods.* Cerenkov counts/min loaded onto each thin-layer plate was 500 cpm. The large and small hollow circles indicate major phosphopeptides identified as CaM Kinase II-specific. Other phosphopeptides that increase are indicated by 'p', while a cross marks a phosphopeptide that decreases, in response to treatment with elevated Ca^{2+} (5 μM).

TLC
B
A
-
+
-
+

FIG. 9. **Two-dimensional tryptic phosphopeptide analyses of MAP-2 phosphorylated *in vitro* by CaM Kinase II and PKA.** Purified MAP-2 was phosphorylated by CaM Kinase IIα (A), or by PKA (B), digested with trypsin and subjected to two-dimensional phosphopeptide mapping as described in *Materials and Methods*. Panel C is the resulting tryptic phosphopeptide map of a mix of A and B. Major phosphopeptides identified as CaM Kinase II-specific are indicated with *arrowheads*; and PKA-specific, with *arrows*. A possible shared site, observed to increase in the comigration map, is indicated by a *plus sign*.


TLC
C
B
A
-
+
-
+
-
+

Lacy *et al.*1972). Dark-field microscopic studies have convincingly demonstrated that secretory granules derived from pancreatic β-cells physically associate with stabilized microtubules through visible link structures which, although not identified, were suggested to be MAPs (Suprenant *et al.*1982). The phosphorylation of MAP-2 by CaM Kinase II and PKA leads, at least *in vitro*, to the increased disassembly of microtubules (Yamamoto *et al.*1985) possibly through microtubule domain "stiffening" as shown for the low molecular weight MAP, tau (Lichtenberg *et al.*1988). The site-specific phosphorylation of MAP-2 by CaM Kinase II could, therefore, regulate the association of secretory granules with microtubules in the β-cell and/or regulate their translocation toward the exocytotic site as a result of changes in microtubule dynamics. Indeed such a role for Ca^{2+}-dependent kinases in granule translocation has recently been obtained from video microscopy experiments in living β-cells (Hisatomi *et al.*1996) and is consistent with recent evidence that this enzyme acts at a site proximal to granule exocytosis (Easom *et al.*1997). These pieces of evidence, combined with recent demonstrations that CaM Kinase II is present in highly purified secretory granule membranes of β-cell insulinoma tissue[1], suggest that this enzyme may be perfectly poised to regulate insulin secretion via the regulation of microtubule function and its association with secretory granules.

[1] Easom, R.A. and Rhodes, C.J. unpublished observations

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CHAPTER IV

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

PREFACE

Molecular events of exocytosis are perhaps best characterized in the neuron. Upon the arrival of an action potential at the nerve terminal, the presynaptic plasma membrane depolarizes, causing a Ca^{2+} influx through voltage-gated Ca^{2+} channels, which triggers the fusion of synaptic vesicles with the plasma membrane and the subsequent release of neurotransmitter. The rapidity of neurotransmitter release has been attributed to the existence of a stable releasable pool of vesicles already docked at the plasma membrane. Evidence has also indicated the presence of a reserve pool of vesicles distal to the exocytotic site that are recruited to the docked releasable pool in response to physiological stimuli. It is thought that, though docked at the active zone, vesicles require further modification prior to becoming competent to fuse with the plasma membrane (Sudhof, 1995), hence the release of only a small portion of the total pre-docked pool per single event. Additionally, the number of vesicles in the releasable pool most likely represents only a small portion of the total number of vesicles. The ability then to regulate the trafficking between the reserve and releasable pools would similarly reflect regulation of neurotransmitter release and efficiency.

Potential candidates for synaptic regulation are proteins associated with synaptic vesicles whose functional role may be modified by phosphorylation. The most likely

candidates are the synapsins, a family of peripherally associated synaptic vesicle proteins that were first discovered as the major phosphoproteins in nerve terminals (Johnson *et al.*1972). Virtually all nerve terminals contain synapsins, synapsin I alone representing approximately 0.4% of the total brain protein, and 9% in a purified synaptic vesicle fraction (Valtorta *et al.*1992a). The synapsins are comprised of four homologous proteins, synapsins Ia/b and IIa/b; the a and b isoforms arising from differential splicing of the primary transcripts of the two genes encoding synapsin I and II (Sudhof, 1990). There is a high degree of homology regarding the amino acid sequence between the synapsins extending from the NH_2-terminus to more than half of each protein, with structural differences due to the splicing, restricted to the COOH-terminal end of the molecule. Accordingly, a suggested model depicts the four synapsins sharing amino-terminal domains (domains A-C), and diverging in their carboxy-terminal domains (Fig. 1). Distinguishing synapsin Ia/b (collectively referred to as synapsin I) from synapsin IIa/b, are the phosphorylation sites in domain D that functionally affect synapsin I. Domain D is also unusual in that it consists of 27% proline, and 17% glutamine, but no asparagine or aromatic amino acids, and is extremely basic due to 17% of its residues being positively charged amino acids.

Thus synapsin I is composed of a globular, hydrophobic head and a proline-rich, elongated, basic tail (Ueda *et al.*1977). Synapsin I binds with high affinity (K_d = 10 nM) and with apparent multiple interactions at distinct sites to phospholipid and protein components of synaptic vesicles (Fig.2). The head region, due to its high surface activity (Ho *et al.*1991), strongly interacts with the acidic phospholipids of the cytoplasmic leaflet of the vesicle membrane and penetrates its hydrophobic core (Benfenati *et al.*1989a). This, however, is not accompanied by membrane destabilization or permeabilization. Indeed, the contrary has been suggested. Synapsin I may stabilize phospholipids in the bilayer, aiding in the maintenance of vesicle integrity and uniform size. In addition, the tight interaction of

FIG. 1. **Domain model of the synapsin family.** The four synapsins are drawn to scale. Taken from Sudhof, T.C. and Jahn, R. 1991 Proteins of synaptic vesicles involved in exocytosis and membrane recycling. Neuron 6: 665-677

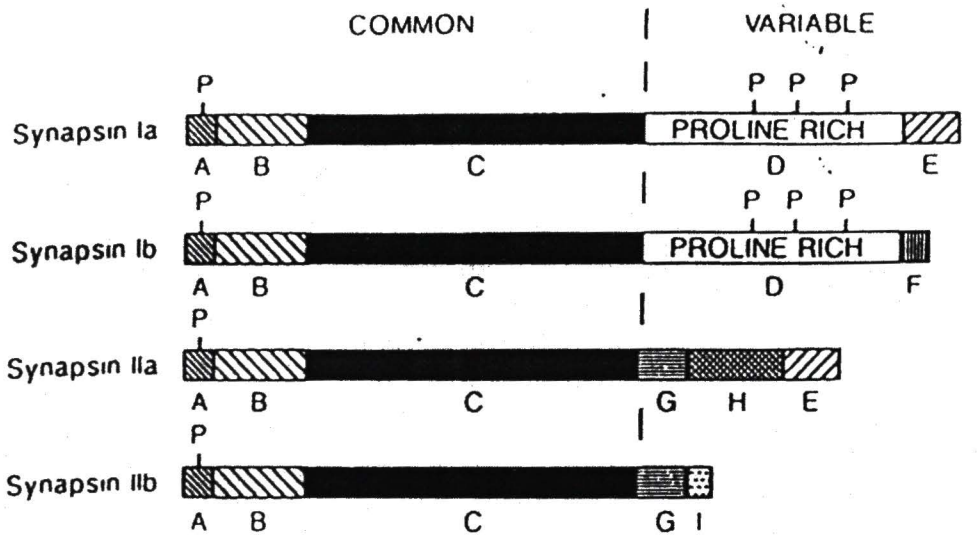
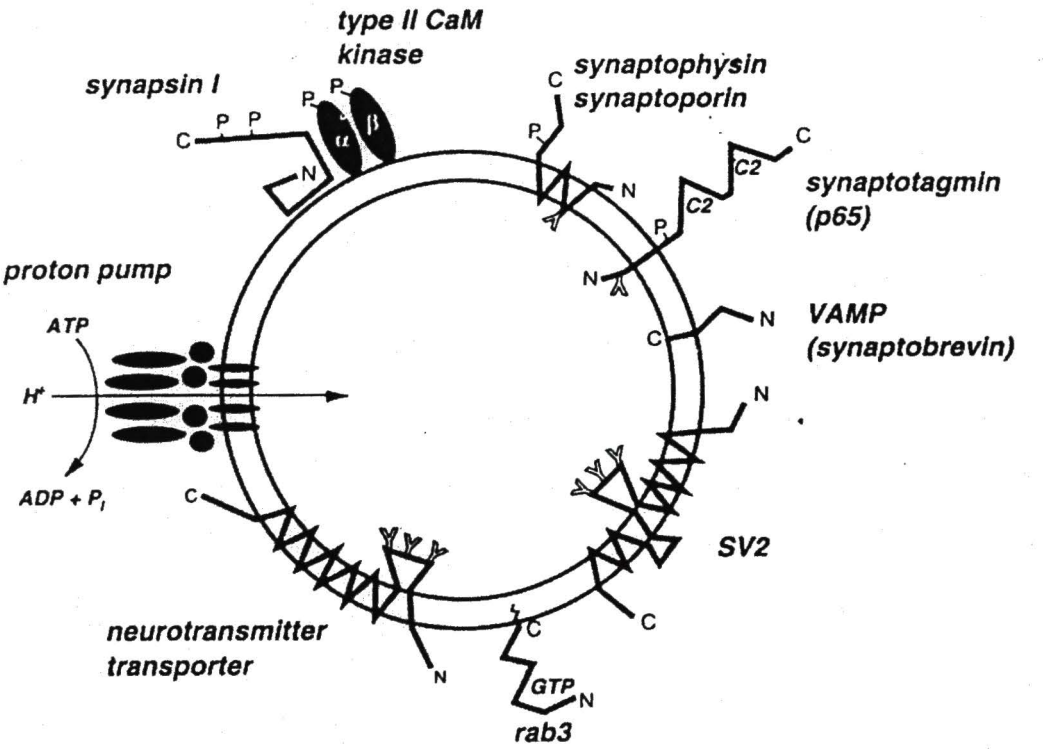

COMMON
VARIABLE
Synapsin Ia
P
P P P
PROLINE RICH
A B C D E
Synapsin Ib
P
P P P
PROLINE RICH
A B C D F
Synapsin IIa
P
A B C G H E
Synapsin IIb
P
A B C G I

FIG. 2. **Components of the synaptic vesicle membrane.** Taken from Bennett, M.K. and Scheller, R.H. 1994 A molecular description of synaptic vesicle membrane trafficking. Annu. Rev. Biochem. 63: 63-100


type II CaM
kinase
synapsin I
synaptophysin
synaptoporin
synaptotagmin
(p65)
proton pump
ATP
H+
ADP + Pi
VAMP
(synaptobrevin)
SV2
neurotransmitter
transporter
GTP
rab3

synapsin I with phospholipids appears to induce vesicle clustering. The synapsin I-induced cross-linking of vesicles may be due to the multiple phospholipid binding sites in one synapsin I molecule or to the strong tendency of synapsin I to self-associate. This data, together with the prompt reversibility of vesicle binding (Benfenati *et al.* 1989a), support more of a surface localization of the head region of synapsin I in binding vesicle phospholipids. The prevention of stronger hydrophobic interactions is also attributed in part to the highly charged tail region. This region of synapsin I specifically binds to a protein component of the vesicles, CaM kinase II, interacting with the regulatory or autoinhibitory domain of the kinase (Benfenati *et al.* 1992b). Though both α- and β-subunits of CaM kinase II can phosphorylate synapsin I and copurify with synaptic vesicles, the binding to synapsin I appears to selectively involve the α-subunit of the synaptic vesicle-associated CaM kinase II (Benfenati *et al.* 1996).

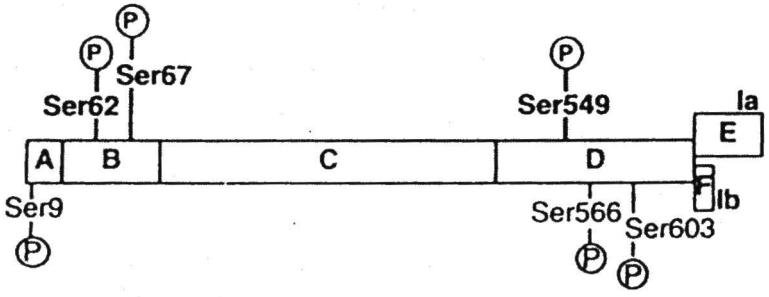
The abundance of synapsin I in the nerve terminal (10 μM) and the projected stoichiometry of 10-30 molecules per vesicle, suggest that synapsin I may cover a large portion of the synaptic vesicle surface, perhaps saturating over 90% of the vesicle sites. While this stabilizes the bilayer, sterically inhibiting contact with Ca^{2+}, paradoxically, the clustering of vesicles by synapsin I enhances both the rate and extent of Ca^{2+}-dependent membrane fusion presumably by decreasing the distance between membranes thereby triggering the hydrophobic contact between the two facing membranes (Allen *et al.* 1990). This aggregating ability of synapsin I, which sequesters synaptic vesicles in the reserve pool, may also prevent the random diffusion of the vesicles from their functional compartment. In fact, functional maturation of neuromuscular synapses caused by the correct compartmentalization of synaptic vesicles was induced by injecting synapsin I into *Xenopus* embryos (Lu *et al.* 1992). Interestingly, immunoelectron microscopic analysis of synaptic vesicle clusters in lamprey reticulospinal axons indicated that synapsin I was predominately associated with the outer portion of the synaptic vesicle cluster but relatively

absent from those vesicles in close proximity to the presynaptic membrane. Moreover, presynaptic injection of synapsin I antibodies resulted in the disappearance of the distal pool and was associated with the marked depression of high-frequency-evoked neurotransmitter release (Pieribone *et al.* 1995). These data strongly implicate the association of synapsin I with the reserve pool of vesicles and its possible role in the regulation of neuroexocytosis.

Synapsin I is also able to bind to several other proteins *in vitro* including grb2, calmodulin, neurofilaments, spectrin, annexin VI, tubulin and actin microfilaments (Sudhof, 1995). Binding to actin filaments leads to bundle formation (Bahler *et al.* 1987) whereas the interaction with actin monomers promotes nucleation and polymerization, affecting the dynamics of actin filament assembly (Benfenati *et al.* 1992a; Ceccaldi *et al.* 1993). Fragment analysis has indicated that the binding sites for synaptic vesicles and for actin are located in distinct regions of the synapsin I molecule (Benfenati *et al.* 1989b). The demonstration of a simultaneous interaction of synapsin I with synaptic vesicles and actin was suggested in quick-frozen, deep-etched nerve terminals and verified by video-enhanced microscopy. Furthermore, the ability of synapsin I to cross-link synaptic vesicle and actin was determined to be specific and not reflective of a non-specific interaction of membranes or simply entrapment of vesicles within the meshwork of actin filaments (Ceccaldi *et al.* 1995).

Initially identified as a major endogenous substrate of cAMP-dependent protein kinase in mammalian brain (Johnson *et al.* 1972), synapsin I currently exhibits six sites of phosphorylation attributable by kinases other than PKA (Fig. 3). All synapsins possess a phosphorylation site for PKA and CaM kinase I at the amino-terminal. However, as previously mentioned, only synapsin I is a physiological substrate for CaM kinase II, which phosphorylates two serine residues in the carboxy-terminal region. Synapsin I exhibits marked conformational changes when phosphorylated by CaM kinase II (Benfenati

FIG. 3. **Phosphorylation sites on synapsin I.** Ser-9 (site 1) is phosphorylated by PKA and CaM kinases I and IV; Ser-566 (site 2) and Ser-603 (site 3) are phosphorylated by CaM kinases II and IV; Ser-62 (site 4), Ser-67 (site 5), and Ser-549 (site 6) are phosphorylated by MAP kinase. Site 6 is also phosphorylated by cyclin-dependent protein kinase1 and 5. Taken from Jovanovic, J.J., Benfenati, S., Siow, Y.L., Sihra, T.S., Sanghera, J.S., Pelech, S.L., Greengard P., Czernik, A.J. 1996 Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions. Proc. Natl. Acad. Sci. USA 93: 3679-3683


P
P
Ser67
Ser62
P
Ser549
Ia
E
A
B
C
D
Ser9
P
Ser566
P
Ser603
P
Ib

et al. 1990) that are associated with major functional alterations of its biological properties, such as modulation of the binding to synaptic vesicles and actin. When CaM kinase II was permitted to phosphorylate synapsin I, the binding between the two and consequently between synapsin I and synaptic vesicles, was practically abolished (Benfenati *et al.* 1996). Therefore the binding of synapsin I to synaptic vesicles not only displays high affinity and saturability, but also sensitivity to phosphorylation of synapsin I by CaM kinase II. The incorporation of the anionic phosphate moiety into the strongly basic carboxy-terminal region was sufficient to disrupt the interactions between the synaptic vesicle-associated kinase and synapsin I indicating the electrostatic nature of their binding and possible involvement of acidic residues within the autoinhibitory domain of CaM kinase II. Alternatively, phosphorylation of synapsin I by its other kinases, such as mitogen-activated protein kinase or PKA, did not significantly affect binding to purified synaptic vesicles (Jovanovic *et al.* 1996).

The ability of synapsin I to initiate and bundle actin filaments is also negatively regulated by phosphorylation by CaM kinase II. Direct visualization of purified components demonstrated that synapsin I reversibly cross-links synaptic vesicles to the actin cytoskeleton under conditions of physiological ionic strength, and that this activity was virtually nullified upon phosphorylation of synapsin I by CaM kinase II (Ceccaldi *et al.* 1995). The reduction of nucleation and consequently formation of actin filaments by CaM kinase II phosphorylation of synapsin I is likely to be physiologically relevant due to the knowledge that the ratio between polymerized and unpolymerized actin strongly influences cytoplasmic viscosity and organelle motility (Fechheimer *et al.* 1993). Therefore, synapsin I phosphorylation by CaM kinase II potentiates the release of synaptic vesicles from the actin cytoskeleton, and due to affecting cytoplasmic viscosity, also prevents their recapture within the actin meshwork.

Increasing evidence suggests that these mechanisms may play a role in regulating neurotransmitter release. Conditions that promote Ca^{2+}-dependent release of neurotransmitter, such as electrical stimulation, or K^+-induced depolarization, also result in increasing the phosphorylation state of synapsin I. Injection of dephosphorylated synapsin I into the squid giant axon (Llinas *et al.*1991), the goldfish Mauthner neuron (Hackett *et al.*1990), or synaptosomes from rat brain (Nichols *et al.*1992), induced a marked reduction of spontaneous and evoked neurotransmitter release. Conversely, the introduction of CaM kinase II into the giant squid axon or rat synaptosomes enhanced neurotransmitter release (Llinas *et al.*1991; Nichols *et al.*1990). Additionally, in an *in situ* study, CaM kinase II-mediated phosphorylation of synapsin I accelerated neurotransmitter release from presynaptic nerve terminals in the hippocampus (Benfenati *et al.*1992b). Studies of mice deficient in synapsin I (Li *et al.*1995b; Rosahl *et al.*1993) or the α-subunit of CaM kinase II (Silva *et al.*1992) demonstrated abnormal paired pulse facilitation, a form of presynaptic plasticity involving availability of synaptic vesicles for exocytosis (Zucker, 1989). The results obtained from these genetic deletion experiments strongly implicate synapsin I in the regulation of synaptic vesicle exocytosis.

These data support the currently accepted model in which the phosphorylation state of synapsin I acts as a switch regulating the interactions between synaptic vesicles and the actin cytoskeleton. Dephosphorylated synapsin I inhibits neurotransmitter release by tethering synaptic vesicles to actin forming a ternary complex. When synapsin I is phosphorylated by CaM kinase II, this complex is disrupted, the inhibitory constraint is relieved, and the reversibly anchored synaptic vesicles are then free to be recruited to the releasable pool. Therefore the synapsin I/CaM kinase II system plays a key role in modulating the trafficking of synaptic vesicles from a reserve pool to a releasable pool, thereby regulating the efficiency of neurotransmitter exocytosis.

In contrast to the neuron, in which vesicles are either already docked at the presynaptic membrane or are tethered by synapsin I to actin filaments in a reserve pool, a resting secretory cell has very few vesicles in contact with or within a short distance (< 150 nm) from the plasma membrane. The majority of vesicles are excluded from release sites by a cortical barrier of actin filaments. Research performed on chromaffin cells, which share a common origin with neurons and common functional features with endocrine cells, has indicated that chromaffin granules are retained within the cortical actin network, apparently associating with actin through short filaments suggested to be anchorage proteins (Trifaro *et al.* 1993).

Collectively, these data describing the involvement of cytoskeletal proteins in the mediation of vesicle transport immediately prior to exocytosis, and the significant effect of the phosphorylation of synapsin I by CaM kinase II on the regulation of neuroexocytosis, lead to the following hypothesis and investigation questioning whether synapsin I functions in a similar manner in acting as an endogenous substrate for CaM kinase II in the pancreatic β-cell.

SUMMARY

Increasing evidence supports a physiological role of CaM kinase II in the secretion of insulin from the pancreatic β-cell but the precise sites of action are not known. In the neuron, this enzyme plays an essential role in neurotransmitter release through the 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 has been studied. Characteristic of neuronal synapsin I, doublet isoforms of Ia/b, were demonstrated in clonal mouse β-cells (βTC3) and primary rat islet β-cells. 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 PKA-specific sites. Immunofluorescence and immunogold electron microscopy verified synapsin I expression in βTC3 cells and pancreatic slices but demonstrated little if any co-localization of synapsin I with insulin containing dense core granules. Thus while this study establishes that synapsin I is a substrate for CaM kinase II in the pancreatic β-cell, it is unlikely that such a phosphorylation event is important for insulin release.

INTRODUCTION

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 (Ashcroft, 1994; Ammala *et al.* 1994; Harrison *et al.* 1984). Concordantly, numerous Ca^{2+}-dependent protein kinases (Hughes *et al.* 1993; Penn *et al.* 1982; Deeney *et al.* 1996; Ganesan *et al.* 1990; Easom *et al.* 1990) and a Ca^{2+}-dependent protein phosphatase, calcineurin (Gagliardino *et al.* 1991), have been identified in various preparations of β-cells but little is known about their endogenous substrates or function. Evidence has recently acumulated that one of these kinases, the mutlifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) may play an important role in insulin secretion. Thus, glucose activates CaM kinase II in isolated rat islets in a concentration-dependent manner (Wenham *et al.* 1994) which temporally correlates with the initial and sustained phases of insulin secretion (Easom *et al.* 1997). Other insulin secretagogues that promote, in part, the mobilization of intracellular Ca^{2+} also activate CaM kinase II (Babb *et al.* 1996). While an attractive candidate for the cellular events associated with exocytosis in the β-cell, a deeper understanding of the physiological role of CaM kinase II can only be attained subsequent to the identification of its endogenous substrates.

Consideration of the mechanism of neurotransmitter release from neurons has provided a model for the functional involvement of CaM kinase II in secretory events. Through the phosphorylation and activation of tyrosine hydroxylase, CaM kinase II is thought to have a global regulatory role to coordinate the rate of neurotransmitter biosynthesis with presynaptic neuron exocytotic activity (Griffith *et al.* 1988; Waymire *et al.* 1988). More mechanistically, CaM kinase II is involved in the distal steps of neurotransmitter release through the phosphorylation of synaptic vesicle-associated proteins

(Greengard *et al.* 1993). Prominent among these is synapsin I which physically associates with CaM kinase II on synaptic vesicles via interactions of C-terminal sequences with the autoinhibitory domain of the enzyme (Benfenati *et al.* 1992b). Under conditions of elevated cytosolic calcium in the activated neuron, the rapid and high stoichiometric phosphorylation of synapsin I at CaM kinase II-specific sites reduces the affinity of synapsin I for vesicle phospholipids and prevents its ability to promote actin nucleating and actin bundling activity (Nestler *et al.* 1998; Ceccaldi *et al.* 1995; Valtorta *et al.* 1992b; Benfenati *et al.* 1990). Through this mechanism, CaM kinase II is thought to effect synaptic vesicle trafficking between a reserve tethered pool and a releasable pool ultimately modulating the availability of synaptic vesicles for exocytosis (Greengard *et al.* 1993).

A number of phenotypic and functional similarities have now emerged between neurons and endocrine β-cells. Both cell types synthesize large dense-core, peptide containing granules as well as smaller, neurotransmitter-containing vesicles, although their prominence in the different cells are reciprocal (Thomas-Reetz *et al.* 1994). The β-cell also expresses an array of exocytotic proteins that correspond to important mediators of neuron secretory events (Wheeler *et al.* 1996) implying that common mechanisms of secretion occur in these cells. This list was recently extended by the observed expression, in clonal β-cells, of microtubule-associated protein-2 (MAP-2) (Krueger *et al.* 1997) and a synapsin I-like protein (Matsumoto *et al.* 1995) previously thought to be largely limited to neurons. Significantly ,and as in the neuron (Maccioni *et al.* 1995), MAP-2 was established to be a substrate for CaM kinase II in β-cells (Krueger *et al.* 1997) implying that this enzyme may also have common functions in both cells. This information has therefore prompted the present study to document expression of synapsin I in clonal and primary β-cells and to assess potential of this protein to serve as a substrate for CaM kinase II.

EXPERIMENTAL PROCEDURES

Materials --- RPMI-1640, glutamine, antibiotics, trypsin/EDTA and fetal bovine serum were purchased from Life Technologies, Inc. Protein A-Sepharose, endoproteinase Glu-C (*Staphylococcus aureus* V8) and α-hemolysin (*Staphylococcus aureus* α-toxin) were purchased from Sigma Chemical Co. Ribonuclease A and TPCK-treated trypsin were acquired from Worthington Biochemical Corp. [γ-^{32}P]ATP was purchased from DuPont NEN. Monoclonal anti-synapsin I (clone 223) was purchased from StressGen Biotechnologies Corp. (Victoria, BC Canada). Anti-synapsin I polyclonal antibody was raised against rat brain synapsin I prepared by the method of Bennett *et al.* (Bennett *et al.* 1983); the resulting anti-sera were purified to an IgG fraction enriched in anti-synapsin I by chromatography on synapsin I-agarose. Mouse recombinant Ca^{2+}/calmodulin protein kinase IIα was generously 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 --- βTC3 cells, obtained from Dr. Shimon Efrat (Albert Einstein College of Medicine, New York), were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 μg/ml penicillin, and 50 μg/ml streptomycin at 37 °C under an atmosphere of 5% CO_2. Permeabilization was achieved by incubation (37 °C for 15 min) with *Staphylococcus aureus* toxin, α-hemolysin, to a concentration of 125-200 U/10^6 cell/0.1 ml Ca^{2+}-free permeabilization buffer (20 mM Hepes, pH 7.0, 140 mM potassium glutamate, 5 mM NaCl, 4 mM $MgSO_4$, 1 mM EGTA and 300 μM Na_2ATP) by a method previously described in detail (Krueger *et al.* 1997).

Cells were resuspended in permeabilization buffer containing 0.05 μM Ca^{2+} and placed on ice prior to experimental treatments. Free Ca^{2+} concentrations in incubation buffers were determined using a Ca^{2+}-electrode (Orion) calibrated against known standards as described by Bers (Bers, 1982).

Immunoblot Analysis --- Immunoblot analyses were performed on nitrocellulose membranes using an ECL™ Western Blotting Analysis System (Amersham; Buckinghamshire, England). Primary and secondary antibody incubations were performed for 1 h each at 25 °C.

Synapsin I Phosphorylation and Immunoprecipitation --- Permeabilized βTC3 cells (approx. 2 x 10^6 per condition) were preincubated at 37 °C for 5 min in 0.05 μM Ca^{2+} permeabilization buffer. The cells were then pelleted and resuspended in 200 μl of 0.05 to 5 μM Ca^{2+} permeabilization buffer with 300 μM [γ-^{32}P]ATP (specific activity, 0.333 Ci/mmole), and incubated at 37 °C for the indicated times. Phosphate incorporation into synapsin I was analyzed by immunoprecipitation procedures as described by Krueger *et al.* (Krueger *et al.* 1997) except SDS polyacrylamide electrophoresis 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 (Krueger *et al.* 1997).

Phosphopeptide Mapping of Synapsin I Following 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 (Huttner *et al.* 1979) with the following modifications; the excised synapsin I band was incubated at room temperature for 30 min in 125 mM Tris-HCl buffer (pH 6.8) containing 0.1% SDS, then placed into wells of a 15% polyacrylamide gel,

overlayed with the same buffer supplemented with 20% glycerol and 4 μg *S. aureus* V8. Subsequent to autoradiography, separation by electrophoresis was conducted at 100V for 20 min, interrupted for 30 min to allow cleavage, and continued at 200V.

Two-dimensional Tryptic Phosphopeptide Mapping of Synapsin I --- For phosphopeptide mapping, ^{32}P-labeled synapsin I was eluted from SDS polyacrylamide gels, and subjected to tryptic digestion as described (Krueger *et al.*1997). Two-dimensional separation of phosphopeptides by electrophoresis and chromatography was performed on a HTLE 7000 thin-layer electrophoresis apparatus (C.B.S. La Jolla, CA) as described (van der Geer *et al.*1994) except that the electrophoresis and chromatography steps were conducted at 1.3 kV for 40 min, and for 14 hr using a phosphochromatography buffer (37.5% n-butanol, 25% pyridine 7.5% glacial acetic acid v/v), respectively.

Immunofluoresence --- βTC3 cells and freshly excised whole rat pancreata were fixed with methanol (-20 °C) or 4% paraformaldehyde, respectively by methods described previously (Lenormand *et al.*1993; Weaver *et al.*1996). Blocking was conducted in phosphate-buffered saline (PBS, 10 mM sodium phosphate, pH 7.2, 150 mM 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 hr at 37 °C in the dark. Repeated washes (3 x 15 min) in PBS containing 0.1% Triton X-100 were conducted between steps. Visualization of slides was conducted on a Nikon Mircophot FXA microscope.

Electron Microscopy --- Rat insulinoma tissue, propagated in NEDH rats (Chick *et al.*1977), was immersion-fixed on ice for 4-6 h with 2% paraformaldehyde/0.2%

glutaraldehyde in 0.1M PBS, pH 7.4, and then infiltrated with 2.3 M sucrose overnight at 4 °C to cryo-protect the tissue. Ultrathin sections (100 μm) were prepared at -95 °C on a Reichert Ultracut S with a FCS cryochamber attachment and collected on Formvar/Carbon coated, glow discharged 200 mesh nickel grids. Following quenching of free aldehydes in the sections by incubation (3 x 5 min) with 0.05M glycine in 0.01M PBS, immunostaining was performed section side down on 20-30 μl droplets in a 60 multiwell chamber. Nonspecific sites were blocked by incubation for 15 min in incubation buffer (10 mM sodium phosphate, 150 mM NaCl pH 7.4, 0.5% BSA, 0.1% gelatin, 20 mM 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 hour each. Control grids were exposed to preimmune serum or ascites fluid as appropriate. The grids were rinsed (6 x 5 min) between each incubation step and (3 x 5 min) after the gold-complex incubation. Sections were finally fixed in 1-2% glutaraldehyde/PBS for 15-20 min, rinsed (2 x 5 min) in PBS, further rinsed in distilled water (5 x 5 min) and reembedded/stained in a 2% aqueous Methyl Cellulose (Sigma:M-6385) containing 0.2% Uranyl Acetate for 10 min. The grids were air dried and viewed on a Zeiss 910 electron microscope (NSF grant #BIR-9413907) at 100kV accelerating voltage. Images were recorded on Kodak SO-163 electron films and processed in a Mohr Pro 8 film/paper processor.

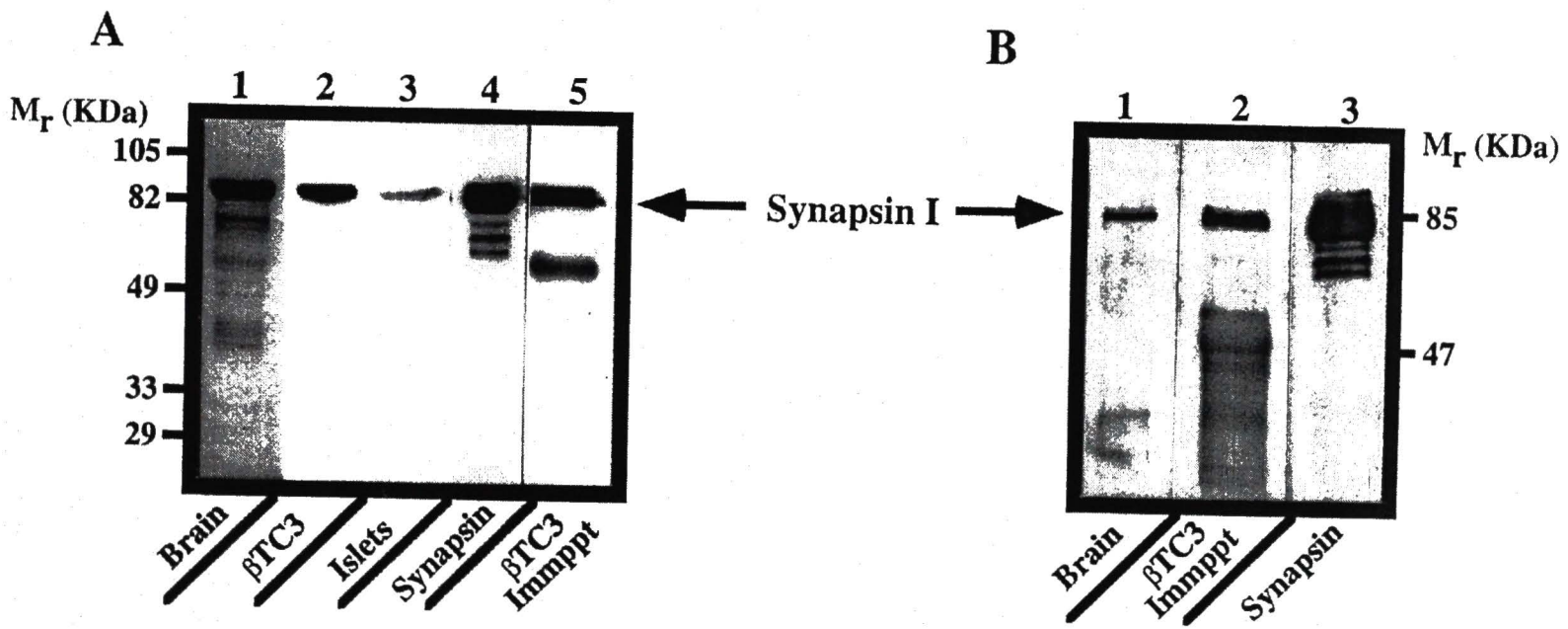
Statistical Analysis --- Data are expressed as the mean ± S.E. determined from at least 3 independent observations unless otherwise stated. Where indicated, statistical significance ($p<0.05$) was assessed by an independent t-test (SAS Institute, Cary, NC).

RESULTS

Expression and Phosphorylation of Synapsin I in βTC3 cells and Pancreatic Islets --- The increasing identification of proteins previously defined as neuron-specific in the pancreatic β-cell supports common biochemical features in neuron and neuro-endocrine cells, particularly with respect to mechanisms of vesicle exocytosis. Despite its prominent role in neuron function, synapsin I has been reported as either absent or expressed only to very low levels in the pancreatic β-cell (Thomas-Reetz *et al.* 1994). Only with the recent demonstration of a synapsin I-like protein in the clonal β-cell line, MIN6 (Matsumoto *et al.* 1995), 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. In these analyses, as well as immunoprecipitates obtained from βTC3 cell lysates (Fig. 4A, lane 5), doublet bands were observed that were reminscent of neuronal synapsin isoforms, Ia and Ib. That this β-cell protein was authentic synapsin I was supported by (i) the recognition of β-cell immunoprecipitates by a monoclonal anti-synapsin I antibody (Fig. 4B), (ii) its *in situ* phosphorylation induced by Ca^{2+} (see below) and (iii) 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 PKA and the Ca^{2+}/calmodulin-dependent kinases I and IV; site 2 (Ser-566) and site 3 (Ser-603) are phosphorylated by CaM kinases II and IV (Czernik *et al.* 1987), and sites 4, 5 and 6 (Ser-62, -67, and -549, respectively) are phosphorylated by mitogen-activated protein kinase (Jovanovic *et al.* 1996). To determine whether synapsin I serves as a physiological substrate for CaM

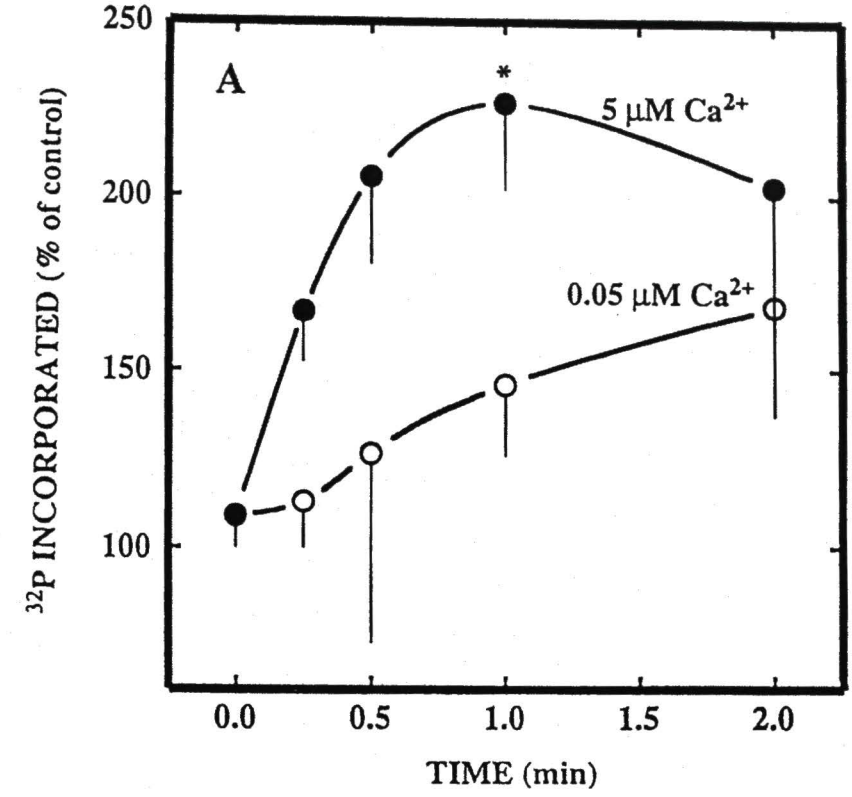
FIG. 4. **Expression of synapsin I in βTC3 cells and isolated rat pancreatic islets.** Immunoblot analyses were performed using rabbit polyclonal (*A*) or mouse monoclonal anti-synapsin antibodies (*B*). *Panel A*: *Lane 1,* whole rat brain homogenate (15 μg protein); *lane 2,* βTC3 homogenate (20 μg 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). *Panel 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).

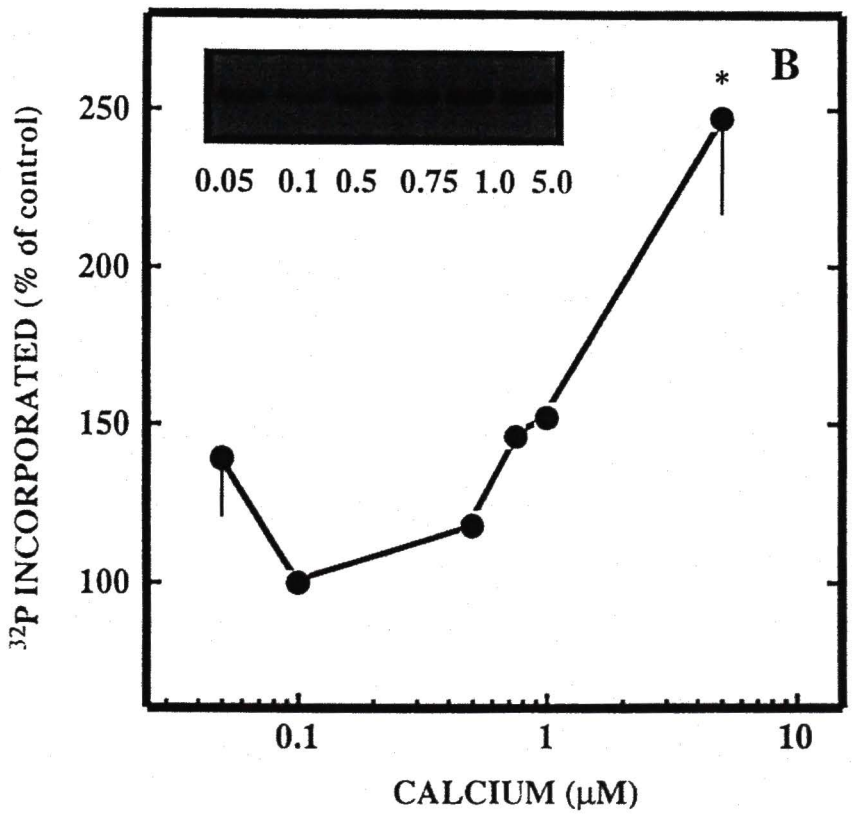

A
B
1
2
3
4
5
M_r (KDa)
105
82
49
33
29
Brain
βTC3
Islets
Synapsin
βTC3 Immppt
Synapsin I
1
2
3
M_r (KDa)
85
47
Brain
βTC3 Immppt
Synapsin

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 (Krueger *et al.*1997), maximal activation of CaM kinase II can be achieved by the elevation of Ca^{2+} from 0.05 μM to 5 μM. In the current study, a similar manipulation significantly increased phosphate incorporation into synapsin I detected by immunoprecipitation and autoradiography. At 0.5 and 1 min, 5 μM 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 μM Ca^{2+} (Fig. 5A). Since 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 exceeding 0.5 μM were necessary to induce significant synapsin I phosphorylation (Fig. 5B). At the maximally effective concentration, 5 μM Ca^{2+}, $^{32}P_i$ incorporation was 208% of control (0.05 μM Ca^{2+}) and half-maximal phosphorylation was achieved at approximately 1 μM Ca^{2+}.

Identification of Site-Specific Phosphorylation of Synapsin I by SV8 Digest and Two-dimensional Phosphopeptide Mapping --- In order 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 approximately 30 and 10 kDa (Fig. 6, lanes 5 and 6). Based on the similarities with digests created from *in vitro* phosphorylation of purified rat brain synapsin I (Fig. 6; lanes 1-4) and previously established criterion (Huttner *et al.*1979), these phosphoproducts harbor site 1 (10 kDa) and site 2 (30 kDa) targeted by PKA and CaM kinase II, respectively. In the presence of

FIG. 5. **Time- and calcium-dependent phosphorylation of synapsin I *in situ*.** Synapsin I was immunoprecipitated from βTC3 cells and phosphate incorporation quantitated by autoradiography and densitometry. *Panel A:* Permeabilized βTC3 cells were incubated in buffers containing free Ca^{2+} concentrations of 0.05 μM (○) or 5 μM (●) at 37 °C for the indicated times. *$p < 0.04$ *vs.* time control (independent *t*-test). *Panel B:* Permeabilized βTC3 cells were stimulated with increasing concentrations of calcium (0.05 - 5 μM) at 37 °C for 1 min. The inset displays an autoradiogram of immunoprecipitated synapsin I phosphorylated at the indicated Ca^{2+} concentrations (μM). * $p < 0.02$ *vs.* 0.05 μM Ca^{2+} (independent *t*-test).


A
*
5 μM Ca2+
0.05 μM Ca2+
250
200
150
100
32P INCORPORATED (% of control)
0.0
0.5
1.0
1.5
2.0
TIME (min)


B
*
0.05 0.1 0.5 0.75 1.0 5.0
250
200
150
100
32P INCORPORATED (% of control)
0.1
1
10
CALCIUM (μM)

basal Ca^{2+} (0.05 μM), phosphorylation of β-cell synapsin I was primarily at site 1, while phosphorylation stimulated by 5 μM Ca^{2+} was observed in both sites though most dramatically at site 2 (Fig. 6). Phosphate incorporation into this site was increased greater than 1.5-fold providing evidence that Ca^{2+} induces the increased phosphorylation of a site distinct from PKA. These results were similar to those observed previously in synaptosomes (Huttner *et al.*1979).

Two-dimensional tryptic phosphopeptide mapping was employed to confirm that Ca^{2+} dependent phosphorylation of synapsin I occurred at CaM kinase II selective sites. For these experiments, βTC3 cells were incubated in the presence of basal (0.05 μM) or stimulatory (5 μM) concentrations of Ca^{2+} and synapsin I then immunoprecipitated and subjected to trypsin digestion. Phosphopeptide maps of synapsin I generated in these conditions were very similar with 6-8 prominent phosphorylation sites evident in both cases (Fig. 7). The elevation of Ca^{2+}, however, resulted in the marked enhancement in the phosphate content of a peptide(s) corresponding to one area of sites indicated by a *plus* sign (Fig. 7B cf. Fig. 7A). Closer analysis of this area (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. 7). Ca^{2+} also induced the increased, albeit less pronounced, phosphorylation of another peptide that was of equivalent net charge but more hydrophobic (Fig. 7).

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 6 major phosphopeptides species (Fig. 8A), three (peptides c, d and e) of which co-migrated with phosphopeptides recovered from β-cell synapsin I (peptides 0, 1 and 2, Fig. 8B & C). The two prominent, acidic

FIG. 6. **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 IIα (*lanes 1 & 2*) or by the catalytic subunit of PKA (*lanes 3 & 4*) as described under "Experimental Procedures." *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 μM (*lane 6*) and 5 μM (*lane 5*). Recovered synapsin was subjected to limited digestion by SV8 protease and to one-dimensional phosphopeptide mapping as described under "Experimental Procedures."

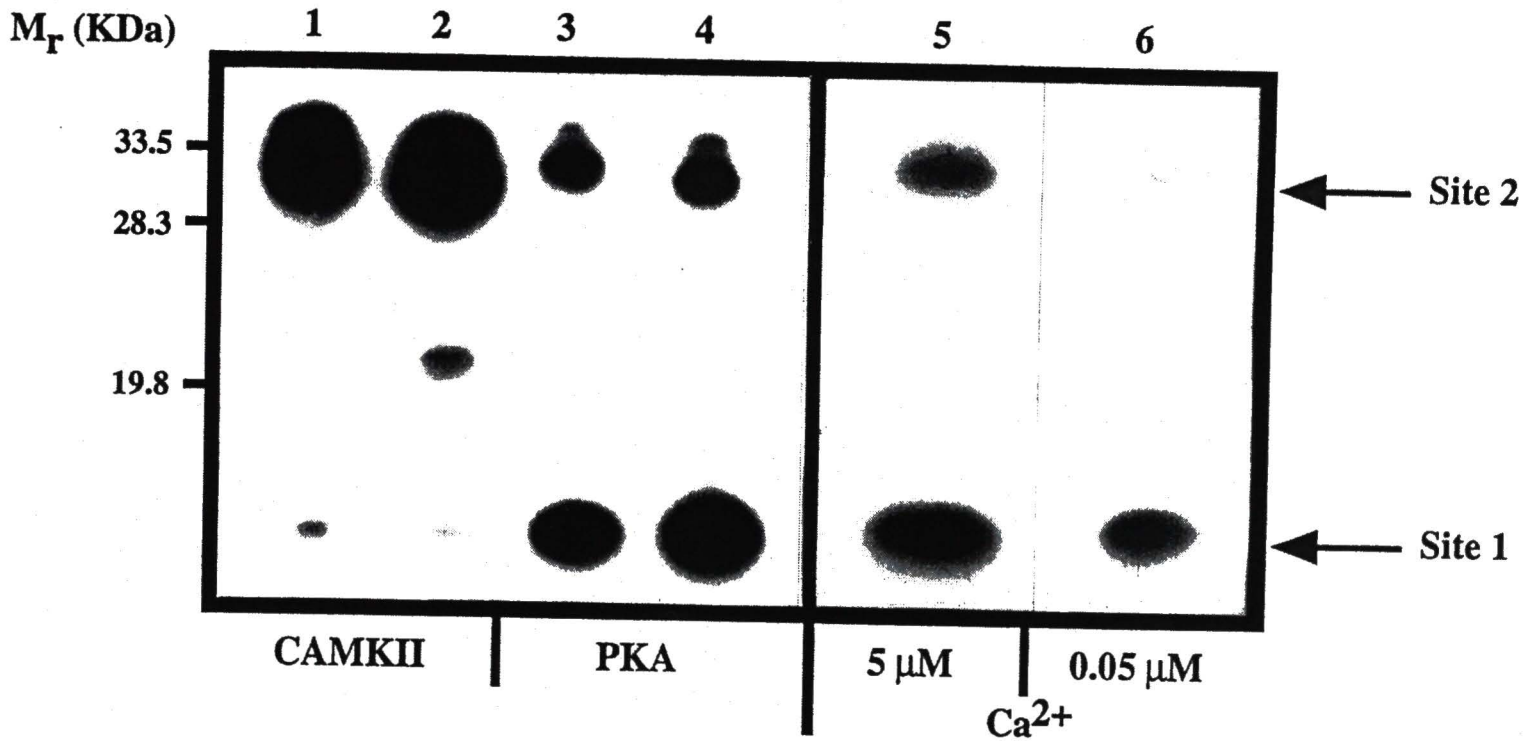

M$_r$ (KDa)
1
2
3
4
5
6
33.5
28.3
19.8
Site 2
Site 1
CAMKII
PKA
5 μM
0.05 μM
Ca^{2+}

FIG. 7. **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 μM (*A*) and 5 μM (*B*), 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 +. Each thin-layer plate was loaded with 265 cpm. (Cerenkov).

TLC
B
A
+
-
+
-

FIG. 8. **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 & C) and synapsin immunoprecipitated from permeabilized βTC3 cells stimulated for 1 min at 37 °C in the presence of 5 μM Ca^{2+} (B & C). *Panel C* represents 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. Cerenkov radioactivity loaded onto thin-layer plates were 2000 cpm (A), 265 cpm (B), and 265 cpm each (C).

TLC
C
B
A
-
+
-
+
-
+

phosphopeptides that were virtually absent from *in situ* maps (Fig. 8 A-B, peptides a and b) may represent N-terminal cyclized pyroglutamyl derivatives of peptides d, e and f (Fig. 8A) as previously identified by Czernik *et al.* (Czernik *et al.* 1987). Of the remaining phosphopeptides visible in *in situ* maps, three peptides, 5, 8 and 9, were likely contributed by the action of endogenous PKA based on their co-migration with peptides generated when synapsin I was phosphorylated by the purified catalytic subunit of PKA (Fig. 9, 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 βTC3 cells, consistent with its potential association with secretory machinery (Fig. 10, A & B). This technique was further employed to ascertain whether the expression of synapsin I was also characteristic of the primary β-cell. Individual islets were identified in frozen sections of rat pancreas through immunofluoresence labeling with anti-insulin antibodies (Fig. 10, E). Islets in subsequent slices were labeled specifically with anti-synapsin I antibody (C) in a manner that could be virtually eliminated by preabsorption of the antibody with purified rat brain synapsin I (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 both expressed synapsin I indicating a distribution throughout core β-cells as well as in mantle α-cells. Immunogold labeling performed in insulinoma tissue or isolated rat islets yielded additional evidence regarding synapsin I localization (Fig. 11). Fig. 11A was representative of the pattern of synapsin I immunoreactivity frequently witnessed.

FIG. 9. **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 μM (B), were digested with trypsin and subjected to two-dimensional ^{32}P-phosphopeptide mapping as described in "Experimental Procedures." *Panel C* is the resulting tryptic phosphopeptide map of the mix of *panels A* and *B*. Cerenkov counts/min loaded for each sample were as follows; (A) 2000 cpm, (B) 265 cpm, and (C) 265 cpm each. Letters label *in vitro* produced phosphopeptides (A) while numbers label *in situ* generated (B). The co-migration map (C) is also accordingly labeled.

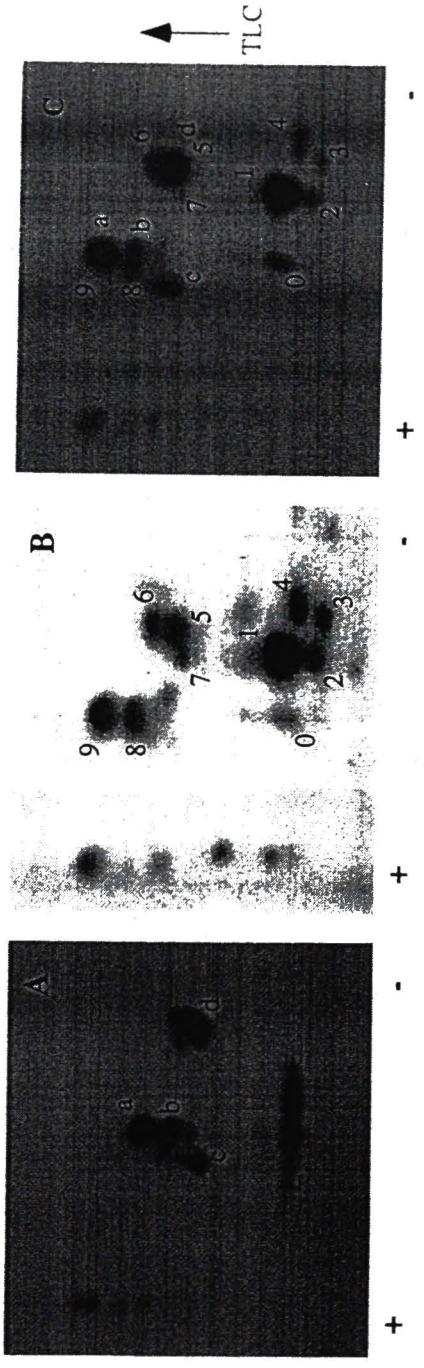

TLC
C
a
b
c
d
e
f
g
h
i
j
B
A
-
+

FIG. 10. **Synapsin I immunoreactivity in βTC3 cells and rat islets in frozen pancreata 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) & (B); βTC3 cells and (C) - (E); rat pancreata. Primary antibodies were: (A) synapsin I; (B) normal rabbit IgG; (C) synapsin I; (D) synapsin I antibody preabsorbed with purified rat brain synapsin I; (E) insulin. Scale bar: A & B = 51 μm, C & D =160 μm, E= 80 μm.

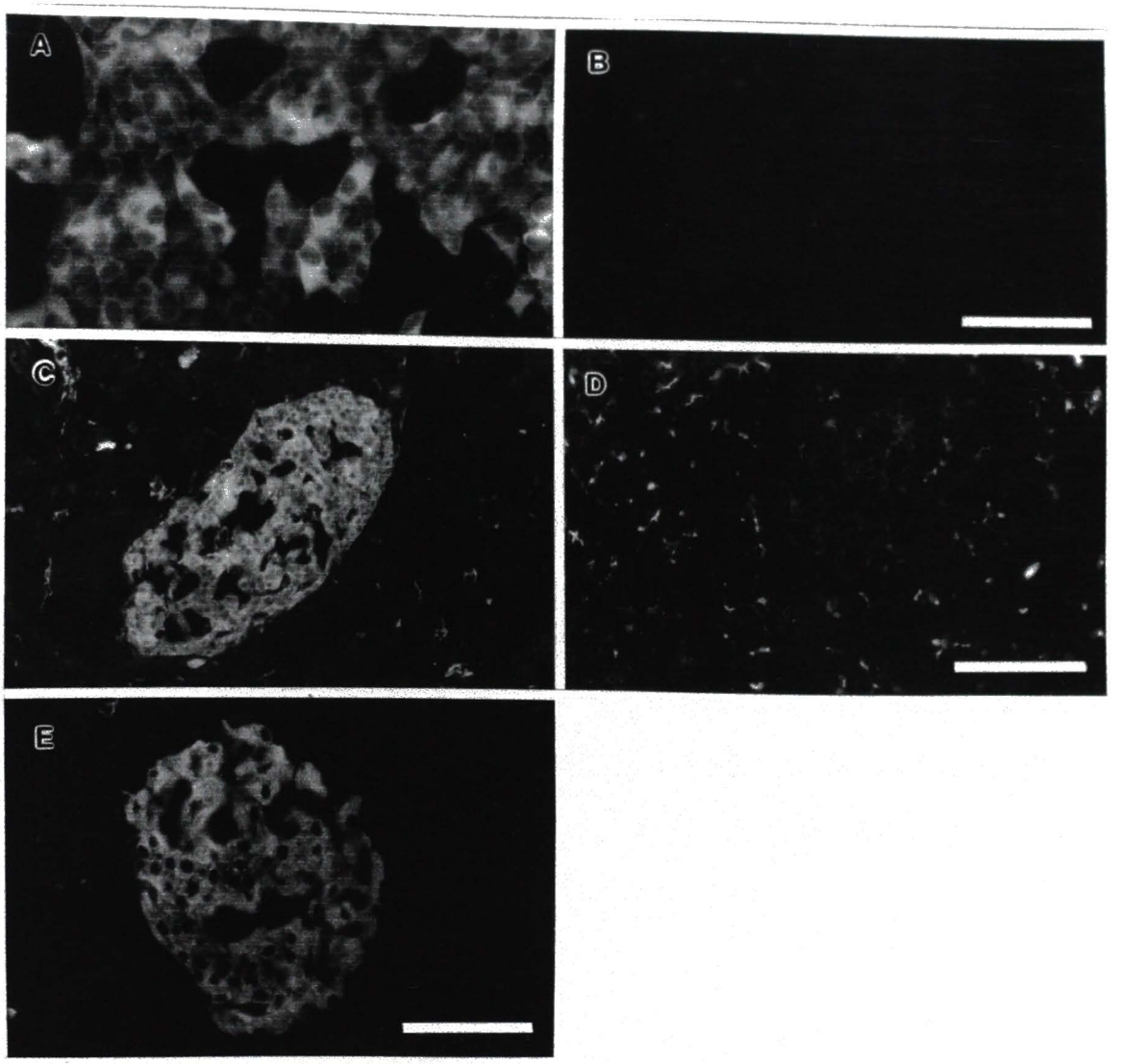
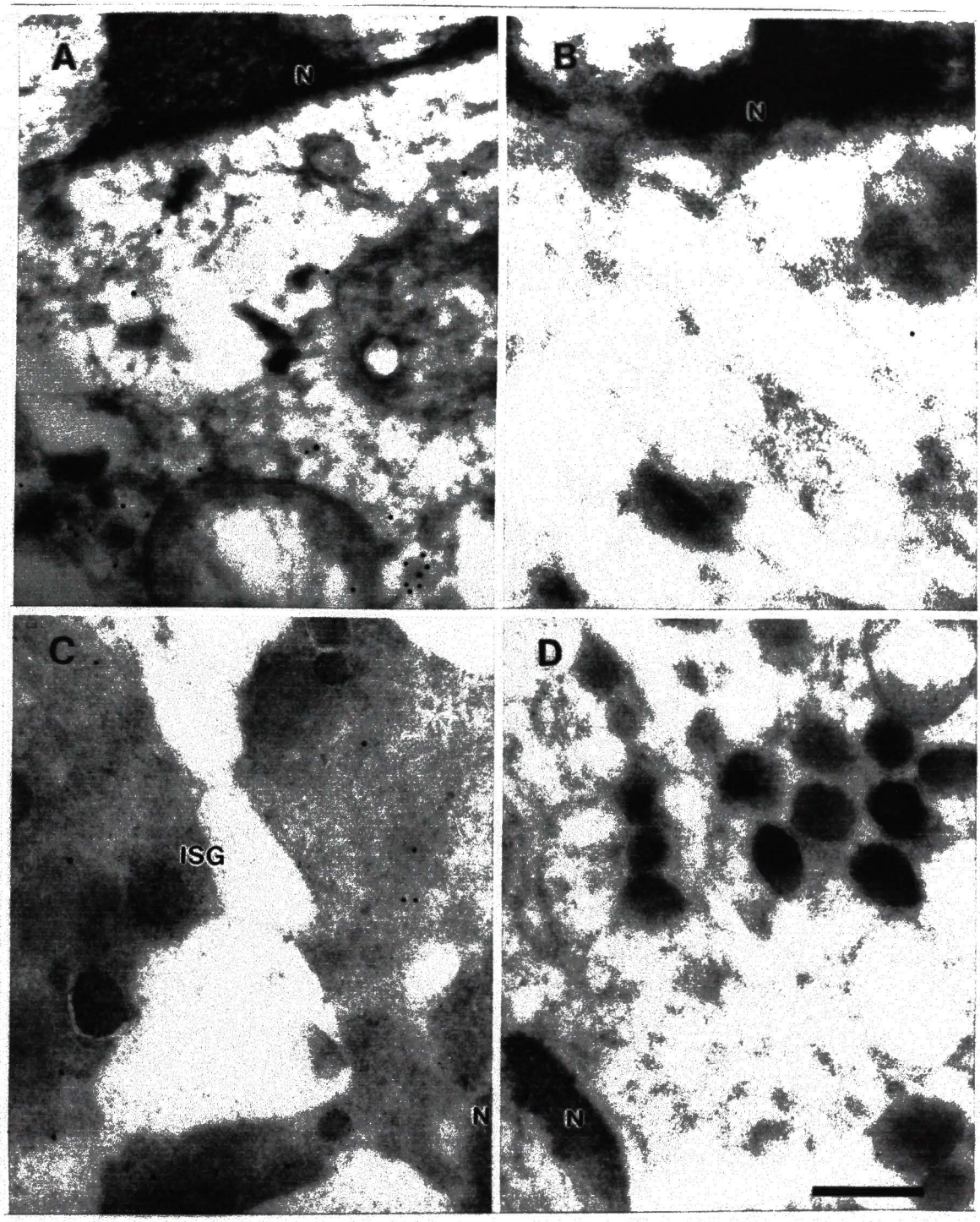

A
B
C
D
E

FIG. 11. **Localization of synapsin I and insulin immunoreactivity in rat insulinoma demonstrated by immunogold labeling.** Cryo-sections of insulinoma were prepared and immunostained as described under "Experimental Procedures." 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 (5 nm)-conjugated anti-mouse antibody. (A) synapsin I (1:100); (B) normal rabbit IgG; (C) synapsin I (1:200) and insulin (1:1000); (D) ascities 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.


A
N
B
N
C
ISG
N
D
N

Noteworthy was the routine observation of the grouping of synapsin I label, apparently associated with vesicle-like organelles of a size inferior to the insulin secretory granule (lower right portion of Fig. 11A). Double labeling studies indicated that while insulin was selectively associated with dense core granules, synapsin I demonstrated little if any co-localization with the same granules (Fig. 11C).

DISCUSSION

Although a common origin for endocrine cells and neurons has been disproved (Teitelman, 1990), the biochemical similarities between the two, specifically with regard to the exocytotic machinery, are mounting. Nonneuronal isoforms of the synaptic exocytotic proteins (SNAREs) have been identified in peripheral tissues such as the lung, liver, spleen, kidney, testis and muscle (Linstedt *et al.*1991; De Camilli *et al.*1990; Bennett *et al.*1993). Additionally, several synaptic vesicle membrane proteins, synaptophysin, synaptotagamin, synaptobrevin, SV2 and rab3A, are expressed in peptide-secreting endocrine cells such as cells of the intermediate and anterior pituitary, adrenal medulla, endocrine pancreas and the parathyroid (Li *et al.*1995a; Schmidle *et al.*1991; Darchen *et al.*1990). Specifically and most recently in islet β-cells, VAMP-2, cellubrevin, SNAP-25, syntaxin isoforms 1-4, and synaptotagmin III have been accordingly identified (Wheeler *et al.*1996). These findings warrant a re-evaluation of what was once termed neuron-specific and a further exploration of more potentially shared attributes between neurons and endocrine cells.

The current study documents the expression of another prominent neuronal protein, synapsin I, in cytoplasmic compartments of the pancreatic β-cell. The appearance of synapsin as a doublet band corresponded with neuronal expression of splice variants of synapsin I, a/b (Sudhof, 1990), an observation that was in contrast to the detection of a single "synapsin I-like" protein (M_r~ 84,000) in MIN-6 cells reported previously (Matsumoto *et al.*1995). Noteworthy, was the prominent expression of the lower band relative to the upper band, reminiscent of the abundant expression of Ib over Ia reported for virtually all synapses (Sudhof *et al.*1991). An important extension here was the detection of synapsin I 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 (Poitout *et al.*1996). 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 (Thomas-Reetz *et al.*1993).

A 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 (Krueger *et al.*1997) and occurred within Ca^{2+} concentrations required to support insulin secretion from permeabilized cells (Easom *et al.*1998). Second, analyses of the sites phosphorylated on synapsin I under conditions of elevated Ca^{2+} were determined as CaM kinase II-specific based on comparisons with phosphorylation patterns generated *in vitro* using purified components. These conclusions were further supported by the close correspondence of the phosphopeptide maps generated in this study with patterns published previously (Czernik *et al.*1987). Thus by one-dimensional SV8 digestion, the most significant Ca^{2+}-induced phosphorylation occurred in the larger fragment (annotated as site 2 in the literature (Huttner *et al.*1979)) corresponding to a C-terminal portion of synapsin I harboring CaM kinase II sites 2 & 3. The modest phosphorylation observed in the smaller fragment is most likely attributable to the presence in the β-cell of a calcium/calmodulin regulated adenylate cyclase (and possibly a cAMP phosphodiesterase)(Sharp, 1979) with subsequent

phosphorylation of site 1 through the activation of PKA. This result was reminiscent of Ca^{2+}-induced phosphorylation of MAP-2 at PKA sites observed under similar conditions (Krueger *et al.* 1997). By two dimensional phosphopeptide mapping, the differential phosphorylation induced by the elevation of Ca^{2+} was accounted for by the phosphorylation of sites that were also targeted by recombinant enzyme *in vitro*. Moreover, these maps were similar to that determined previously for CaM kinase II phosphorylated synapsin I from rat and bovine brain which generally located sites 2 and 3 in the lower half of the map (Czernik *et al.* 1987). Differences observed between maps generated from synapsin I immunoprecipitated from βTC3 cells and purified rat brain were not surprising, based on the previous determination of peptide cyclization (Czernik *et al.* 1987) and the different environments of the two systems in presentation of the substrate to the kinase.

The phosphorylation of synapsin I by CaM kinase II in neurons is a critical step in the release of neurotransmitter from presynaptic terminals by releasing tethered vesicles. By extrapolation, this defined mechanism suggests that the activation of β-cell CaM kinase II may facilitate vesicle exocytosis through phosphorylation of synapsin I in β-cells. The shunting of insulin containing secretory granules to specified compartments of the β-cell is facilitated by transient association with cytoskeletal structures (Malaisse *et al.* 1975; Dentler *et al.* 1986). However, despite a previous suggestion of a synapsin I-like protein in isolated preparations of insulin secretory granules (Matsumoto *et al.* 1995), no evidence could be obtained for the localization of synapsin with insulin granules by immunogold labeling on fixed sections of β-cell insulinoma. Thus, while synapsin I was phosphorylated by CaM kinase II in conditions favoring insulin secretion, these data do not support a role for this event in the preparation of insulin granules for exocytosis. However, the apparent grouping of gold particles directed to synapsin I to small faintly defined structures observed in electron microscopy suggest that synapsin I may be associated to other vesicles in the β-cell. In this respect, it is of interest that the β-cell possesses synaptic-like microvesicles that

are considerably smaller (~50 nm) than insulin secretory granules (~70-200 nm) that are responsible for the packaging and transport of the neurotransmitter, GABA (Thomas-Reetz *et al.* 1994; Reetz *et al.* 1991). Like insulin secretion, GABA release appears to be regulated under conditions, such as elevated concentrations of glucose, that increase cytosolic calcium and induce the activation of CaM kinase II (Gaskins *et al.* 1998) leaving open a possible role of synapsin I phosphorylation in the release of this neurotransmitter from β-cells. It may be of future importance, therefore, to address the potential association of synapsin I with synaptic-like microvesicles in the β-cell.

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CHAPTER V

PHYSIOLOGICAL RELEVANCE FOR MAP-2 PHOSPHORYLATION

INTRODUCTION

Comprehension of the role of CaM kinase II in the mediation of insulin secretion can only be attained subsequent to the identification of its endogenous substrates. A major objective of these investigations was to evaluate whether MAP-2 and synapsin I serve as endogenous substrates for CaM kinase II in the pancreatic β-cell. Though convincingly accomplished, not directly addressed was the functional effect on insulin secretion due to the phosphorylation of MAP-2 or synapsin I by CaM kinase II. Thus, while the activation profile of CaM kinase II temporally parallels that of insulin secretion and the phosphorylation of MAP-2 and synapsin I, evidence supporting a major role by CaM kinase II in glucose-induced insulin secretion is still indirect and circumstantial. Because synapsin I immunolocalization was distinct from insulin secretory granules, continuing research explored the potential of MAP-2 regulation by CaM kinase II impacting the secretion of insulin.

An important extension of these previous investigations will be to conduct future studies in primary β-cells, thereby eliminating the possibility of artifactual results induced from the immortalization of β-cell long-term cultures. Accordingly, the determination of the physiological significance of the phosphorylation of MAP-2 would require demonstrations of its regulation by insulin secretagogues (i.e.; glucose) in the primary β-cell. Initially pertinent, again however, the association between the two events, MAP-2 phosphorylation and insulin secretion, could only be suggestive and tenuous. The underlying hypothesis

propelling this dissertation proposes that an activated CaM kinase II via phosphorylation of its endogenous substrates plays a major role in orchestrating intermediate mechanisms culminating in insulin exocytosis. This implies that phosphorylation of MAP-2 by CaM kinase II is required for insulin secretion. Ultimately then, to establish the relevance between these events, the phosphorylated site/s on MAP-2 specific to CaM kinase II during glucose-induction of the primary β-cell must be identified and sequenced. Once established, site-directed mutagenesis converting phosphoryl acceptor sites to alanyl residues of the cDNA coding for MAP-2 can be performed. After successful transduction of βTC3 cells, pancreatic β-cells may then be transduced by a recombinant adenovirus containing wild-type or mutant MAP-2 cDNA, and functional assays measuring the effects of the mutation could be conducted (i.e.; perifuse the islets and assay for secreted insulin).

The position of a phosphate moiety at a site on MAP-2 recognized by a specific kinase is thought to impart functional differentiation. However, the regulatory role of the differential phosphorylation of MAP-2 *in vivo* remains to be clarified. Identification of CaM kinase II-specific sites on MAP-2 for potential manipulation would aid in the detailed study of MAP-2 as well as provide some insight regarding the role of CaM kinase II in the pancreatic β-cell. The following experiments and results reflect the initial efforts in addressing these issues.

RESULTS AND CONCLUSIONS

Immunofluoresence --- Previously, immunoblot analyses had indicated that isolated rat islets express MAP-2 and that islet immunoprecipitable MAP-2 was also immunoreactive. Therefore, immunofluoresence was performed to examine the cellular distribution of MAP-2 in βTC3 cells and isolated islets (Fig. 1). βTC3 cells and freshly excised whole rat pancreata were prepared as described (Lenormand *et al.* 1993; Weaver *et al.* 1996). MAP-2 immunoreactive puncta appeared diffusely dispersed throughout the cytoplasm of βTC3 cells, consistent with its association with microtubules. To ascertain whether MAP-2 expression was also characteristic of the primary β-cell, this technique was repeated with frozen sections of rat pancreas. Double labeling studies that identified individual islets by use of anti-insulin antibodies, described the prevalent expression of MAP-2 largely co-localized with insulin (Fig. 1C). Closer inspection of this figure revealed the extension of MAP-2 expression into the β-cell mantle, beyond the core insulin-identified β-cells. Interestingly, in double labeling studies not shown, MAP-2 immunoreactivity was also observed in the β-cell mantle, specifically in glucagon secreting α-cells.

Double labeling studies were also conducted examining the immunolocalization of CaM kinase II in comparison to MAP-2 (Fig. 2) and insulin (Fig. 3). These data suggest an association between CaM kinase II, insulin, and MAP-2, though the relative fluoresence for CaM kinase II appeared diminished. This was most likely due to the lack of antibody avidity also witnessed in immunoblots with the same anti-CaM kinase II antibody. Future studies employing immunogold electron microscopy will most likely yield additional evidence regarding the localization and associations between CaM kinase II, MAP-2 and insulin.

FIG. 1. **MAP-2 immunoreactivity in βTC3 cells and rat islets in frozen panreata slices.** MAP-2 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. Primary antibodies were: (A) MAP-2 in βTC3 cells; (B) MAP-2 in rat pancreata; (C) MAP-2 and insulin in rat pancreata. Yellow indicates co-localization. Scale bar: A = 51 μm, B = 80 μm, C = 100 μm.

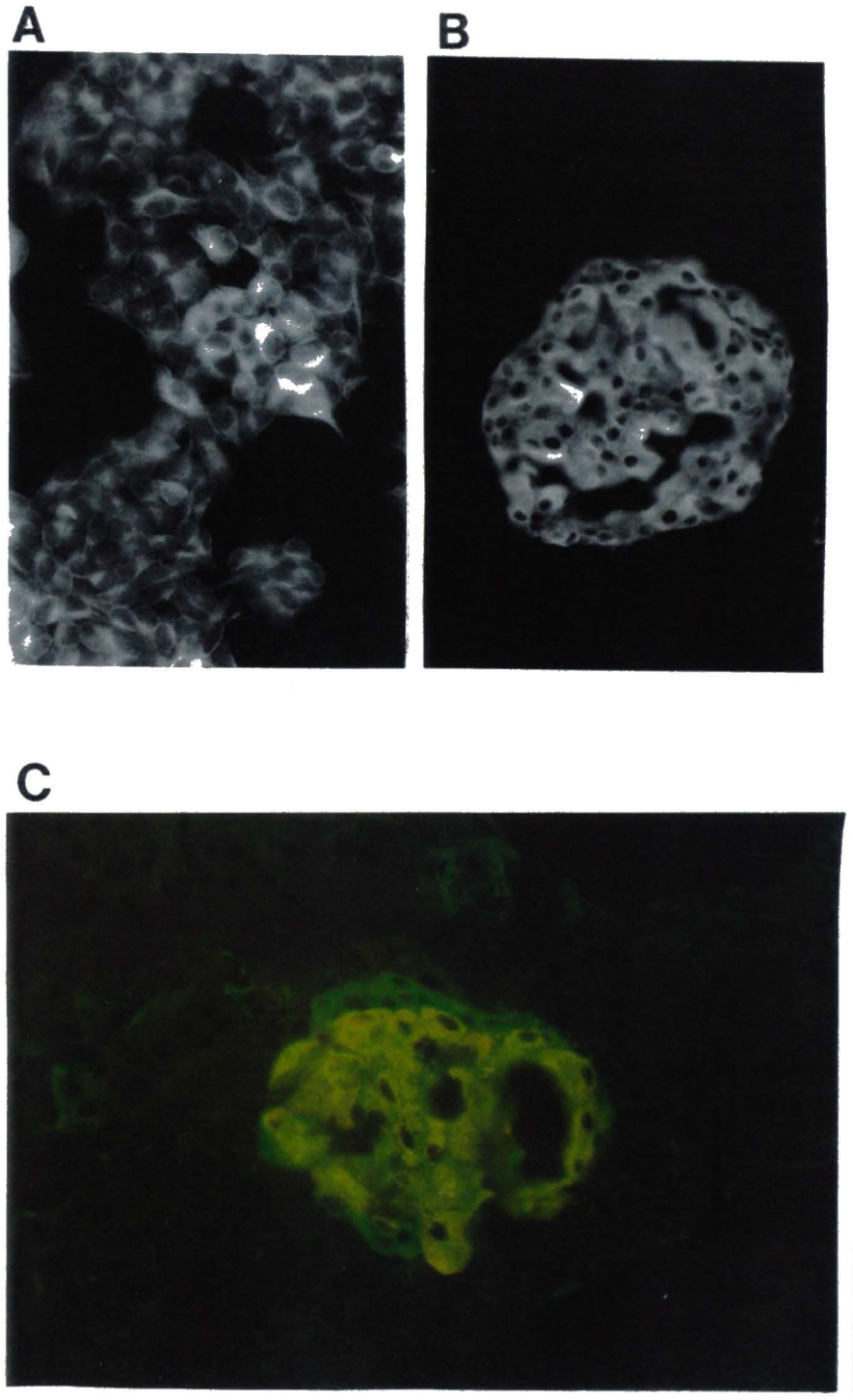
A
B
C

FIG. 2. **CaM kinase II and MAP-2 immunoreactivity in rat islets in frozen pancreata slices.** MAP-2 and CaM kinase II were detected by indirect immunofluorescence in rat pancreata. Visualization was accomplished with a fluorescein-conjugated anti-rabbit antibody for CaM kinase II and a Tx-Red X-conjugated anti-rabbit antibody for MAP-2. Primary antibodies were: (A) MAP-2; (B) CaM kinase II; (C) Both MAP-2 and CaM kinase II. Orange indicates co-localization. Scale bar: A and B = 160 μm, C = 100 μm.

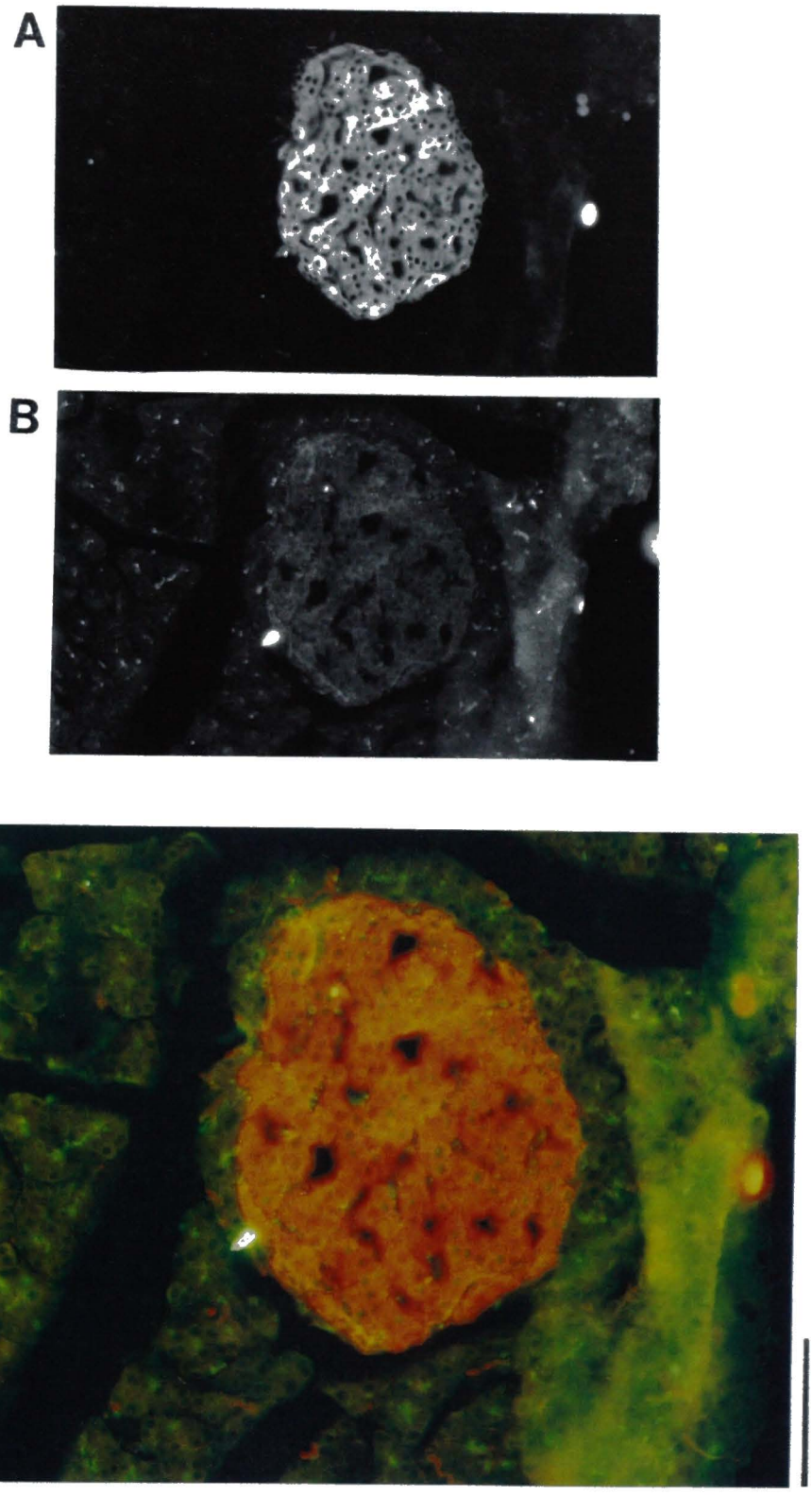
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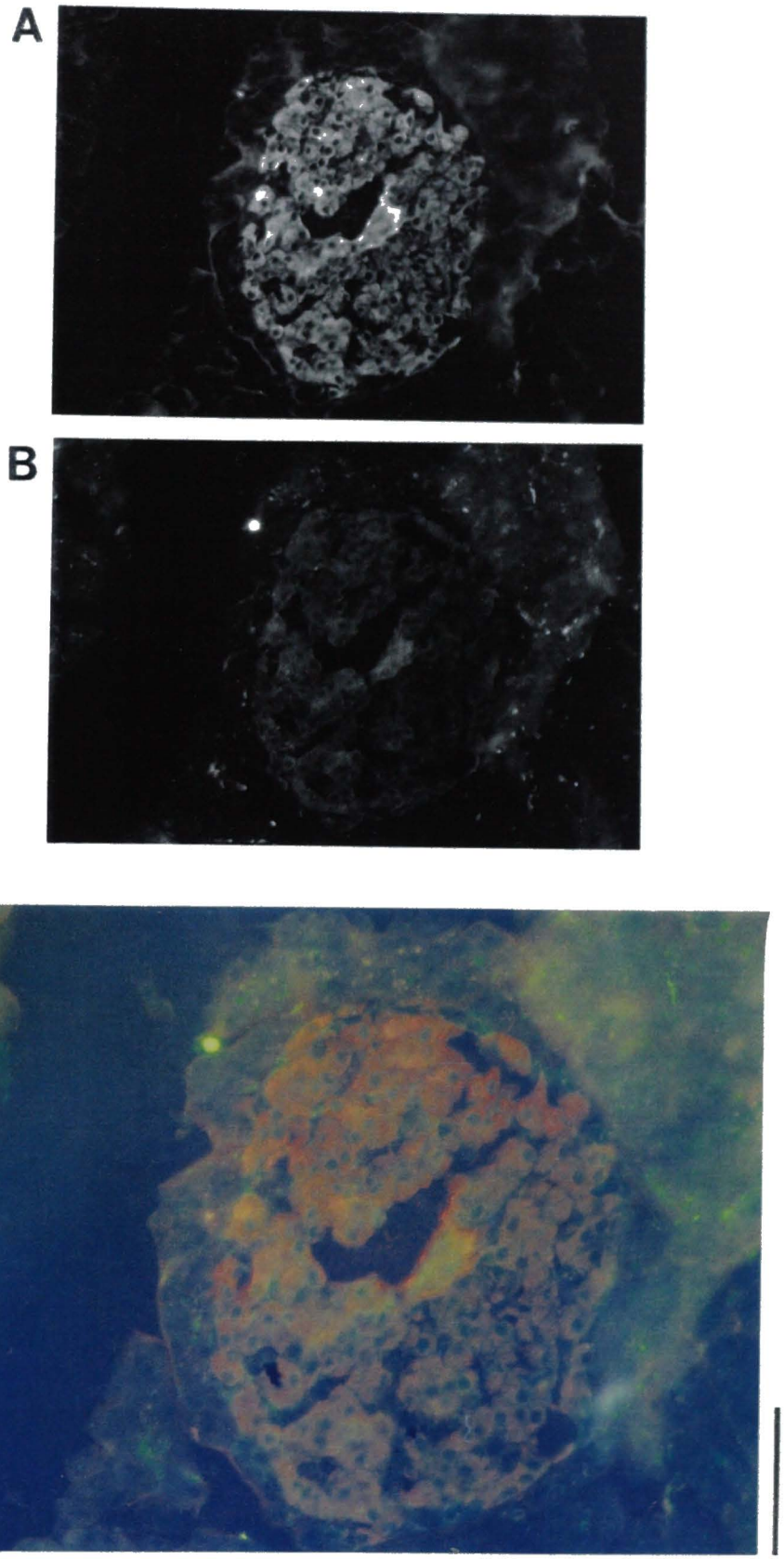
FIG. 3. **CaM kinase II and insulin immunoreactivity in rat islets in frozen pancreata slices.** Indirect immunofluorescence aided in the detection of CaM kinase II and insulin in rat pancreata. CaM kinase II was labeled by a fluorescein-conjugated anti-rabbit antibody, while a rhodamine-conjugated anti-mouse antibody labeled insulin. Primary antibodies were: (A) insulin; (B) CaM kinase II; (C) Both insulin and CaM kinase II. Orange/yellow indicates co-localization. Scale bar: A and B = 128 μm, C = 80 μm.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C

Insulin Secretion --- Prompting the next experiment, were the investigations of inhibition of secretion by the drugs, estramustine and its derivative, estramustine-phosphate, in which the interaction with MAP-2 was described as the possible mechanism of drug action. Estramustine, an adduct of estradiol and nor-nitrogen mustard, is an antineoplastic drug used in the treatment of prostatic carcinoma, clinically administered as the phosphorylated analogue, estramustine phosphate (EP) (Pedrotti *et al.*1997). These drugs have been demonstrated to block secretion and to interfer with microtubule assembly, effects mediated by the drugs' interaction with MAP-2 (Stearns *et al.*1991; Martinez *et al.*1992). Structural analyses revealed that the drugs bound to the tubulin binding domains at the carboxy-terminal region of MAP-2. Interestingly estramustine phosphate, not estramustine, blocked MAP-2-induced microtubule assembly, indicating that the negatively charged phosphate moiety was of importance for the inhibitory effect (Moraga *et al.*1992). Similarily, estramustine phosphate, but not estramustine, inhibited the interaction of MAP-2 with actin filaments (Pedrotti *et al.*1997). Taken together, these data suggest that these anti-mitotic agents may block secretion by a mechanism involving interactions with MAP-2.

Thus, a plausible hypothesis to test would be that treatment of isolated islets by estramustine phosphate would result in the inhibition of the secretion of insulin. A perifusion procedure would be the most appropriate technique to examine this relationship. Following incubation (2 hr) in the presence or absence of the drug (estramustine or estramustine phosphate), isolated islets would be placed on hydrophilic 13-mm polycarbonate cyclopore filters in Swinnex chambers and perifused wtih KRB basal medium (3 mM glucose) in the presence or absence of the drug at 1 ml/min for 30 min at 37 °C. The perifusion would then continue with either the same medium or KRB medium containing 17 mM glucose, again with or without the drug. The perifusate would be collected at specific times and analyzed for insulin content.

The pilot experiment demonstrated promising results in support of the hypothesis. Attenuation of glucose-induced insulin secretion from islets in the presence of estramustine phosphate was indicated, however, the amount of insulin released by glucose-induced islets in the absence of the drug was uncharacteristically diminished relative to past data. These experiments warrant repeating for a more accurate assessment of the effect of estramustine and estramustine phosphate on insulin secretion.

Development of Back Phosphorylation Technique --- While the possible outcome of estramustine phosphate causing inhibition of insulin secretion is intriguing, and while estramustine phosphate appears to compete with tubulin for binding to MAP-2 thereby interfering with the ability of MAP-2 to interact with microtubules, an effect also mimicked by an *in vitro* selectively phosphorylated MAP-2, the association between MAP-2 and the exocytotic event remains indirect. A direct link between the two events, MAP-2 phosphorylation and insulin secretion, demand that demonstrations of the differential phosphorylation of MAP-2 at CaM kinase II-specific sites correlate with, and effect, glucose-induced insulin secretion from isolated islets. To begin the identification and isolation of these sites, subsequent to the expression of various mutants in islets, a unique strategy was proposed. In brief, the back phosphorylation technique refers to the phosphorylation of pancreatic β-cell MAP-2 by purified recombinant CaM kinase II subsequent to islet perifusion. The working postulate of MAP-2 phosphorylation by CaM kinase II concomitant with glucose-induced insulin secretion provided the foundation for this technique. After perifusing islets with either basal or stimulatory concentrations of glucose, islet MAP-2 would be isolated and incubated with [γ-^{32}P]ATP and recombinant CaM kinase II. Theoretically, the radiolabeled phosphate would be incorporated at vacant CaM kinase II sites on MAP-2. Presumably then, basal conditions in which CaM kinase II is inactive, will later reveal greater phosphate incorporation onto MAP-2 in contrast to

glucose-stimulated conditions. Comparison of two dimensional phosphopeptide maps might then reveal the few CaM kinase II-specific sites that are phosphorylated under physiologically stimulating conditions.

Characterization of the final phase of the procedure, the phosphorylation of immunoprecipitated MAP-2 by exogenous CaM kinase II, was first targeted. Initial approaches in optimizing reaction conditions *in vitro* and with βTC3 cells were problematic. To isolate MAP-2 by immunoprecipitation, protein A-sepharose was selected for the solid phase. However, upon testing *in vitro* the effect of each purified component sequentially, the addition of protein A-sepharose resulted in loss of signal from MAP-2. It was suggested that either protein A-sepharose interfers with the ability of CaM kinase II to phosphorylate MAP-2, and/or that it adsorbs the kinase. Interestingly, the addition of the MAP-2 antibody appeared to improve the ability of MAP-2 to be phosphorylated by CaM kinase II, perhaps in providing a better presentation of the substrate to the kinase. An alternative approach to immobilize the antibody by ELISA (enzyme linked immunosorbance assay) was also unsuccessful due to the inability to recover MAP-2. Utilizing protein A again, it was reasoned that relying on the thermostable properties of MAP-2, separation of MAP-2 and its antibody from protein A prior to kinase addition could be accomplished by boiling and centrifugation. Though apparently successful *in vitro* (Fig. 4), this procedure when duplicated in βTC3 cells and islets resulted in an absent ^{32}P-labeled MAP-2. Immunoblot analysis of the various fractions aliquoted at distinct steps throughout the procedure revealed that while the antibody-protein A complex was efficiently capturing MAP-2 from the cellular lysate, boiling did not release MAP-2 from this complex to the supernate to be phosphorylated by exogenous CaM kinase II (Fig 5).

To circumvent the difficulty of recovering MAP-2 from the antibody-protein A complex, phosphorylation of MAP-2 by exogenously added CaM kinase II prior to MAP-2 immunoprecipitation was proposed. Starting with viable tissue, slight sonification to

FIG. 4. **The *in vitro* phosphorylation of MAP-2 by CaM kinase II.** After incubation of MAP-2 (*lane 1*), MAP-2 with antibody (*lane 2*), or MAP-2 with antibody/protein A complex (*lane 3*), samples were supplemented with 5 mM DTT and 50 mM PIPES buffer, boiled, centrifuged and the supernatant transferred to a clean tube. Subsequent to the addition of CaM kinase II and activators (described in Chapter III), phosphorylation proceeded for 1 min, was terminated, and samples were subjected to SDS-polyacrylamide electrophoresis on a 5% gel. Shown is the resulting autoradiogram.

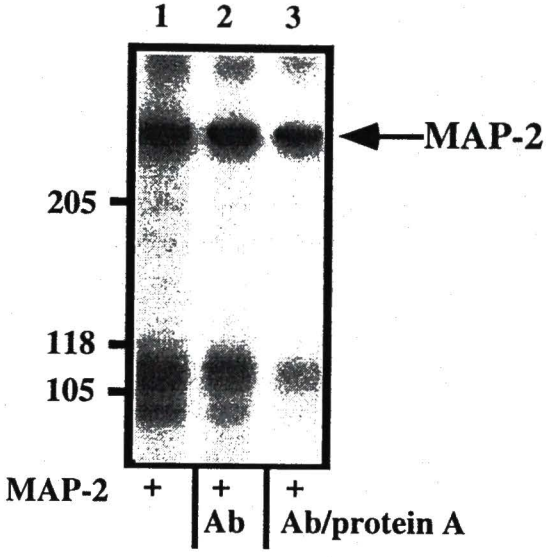
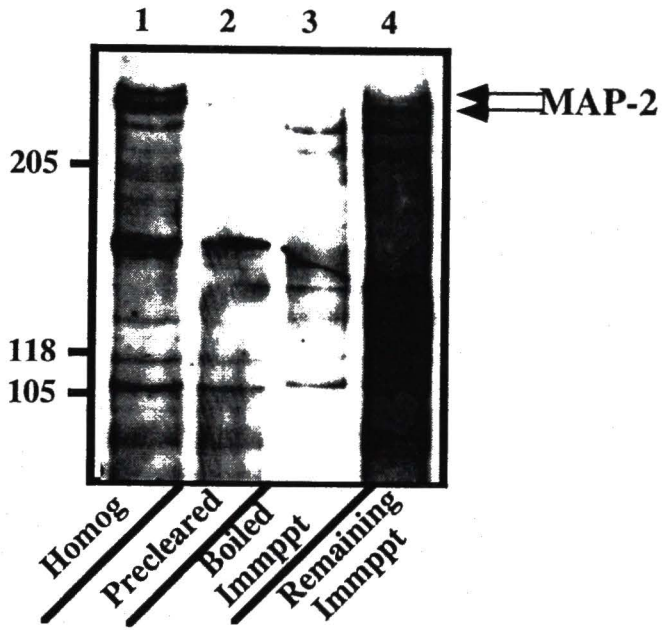

1
2
3
MAP-2
205
118
105
MAP-2
+
+
Ab
+
Ab/protein A

FIG. 5. **Expression of MAP-2 in various fractions throughout the back phosphorylation protocol.** Immunoblot analyses were performed using a rabbit anti-MAP-2 antibody at various stages of MAP-2 immunoprecipitation and back phosphorylation from isolated rat islets (~ 300). *Lanes 1* and *2*, islet homogenate before and after addition of protein A-sepharose; *lane 3*, clarified supernatant following boiling of immunoprecipitation pellet; *lane 4*, remaining protein A-sepharose immunoprecipitate.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disrupt cellular membranes was necessary to enable the exogenous kinase access to its endogenous substrates. Heat inactivation of endogenous proteins prior to CaM kinase II addition was deemed prudent. Finally, accommodating the requirements for optimum activation of CaM kinase II was also a consideration. Though initially promising when conducted with βTC3 cells, with islets, this protocol too was riddled with complications, beginning again with the loss of ^{32}P-labeled MAP-2. While successful *in vitro* experiments were conducted in tandem with islets reassuring validity of the protocol, the inclusion of islet homogenate noticeably attenuated relative phosphate incorporation into CaM kinase II ostensibly effecting its activation. To ascertain whether this inhibition was due to components of the islet extract or was an issue of sensitivity, exogenous MAP-2 was added simultaneously with CaM kinase II. Exogenous MAP-2 was phosphorylated in the presence of islet lysate, therefore indicating a sensitivity problem (Fig. 6). Noteworthy was the marked attenuation of phosphorylation in native versus heat inactivated samples, possible reflective of active phosphatases. Due to possible competition for [γ-^{32}P]ATP, buffer adjustments were made and differential MAP-2 phosphorylation between basal- and glucose-stimulated islets was observed (Fig. 7). Supplementing with microcystine, a phosphatase inhibitor, was ineffectual. Efforts to reproduce this first observed ^{32}P-labeled MAP-2 differential however, were unsuccessful. Closer scrutiny of the stimulated islet sample in Fig. 7 reveals suspicious generalized reduction of phosphorylation rather than specifically for MAP-2. Moreover, though exogenously added MAP-2 was phosphorylated by CaM kinase II in the presence of cellular lysate, absence of lysate resulted in the marked enhancement of phosphate incorporation into MAP-2 and CaM kinase II, suggestive of some inhibitory element within the lysate. Returning to isolating β-cell MAP-2 first for later presentation of CaM kinase II will likely be more productive. To this end, future efforts employing antibody linked magnetic beads are forthcoming.

FIG. 6. **Comparison of the back phosphorylation of endogenous and exogenous MAP-2 by CaM kinase II.** Exogenous CaM kinase II, in the presence (*lanes 2* and *4*) or absence (*lanes 1* and *3*) of exogenous MAP-2, was added to rat islet homogenate either heat inactivated (*lanes 1* and *2*) or not (*lanes 3* and *4*). MAP-2 was then immunoprecipitated and phosphate incorporation determined by autoradiography. *Lanes 5* and *6* represent purified components without islet lysate.

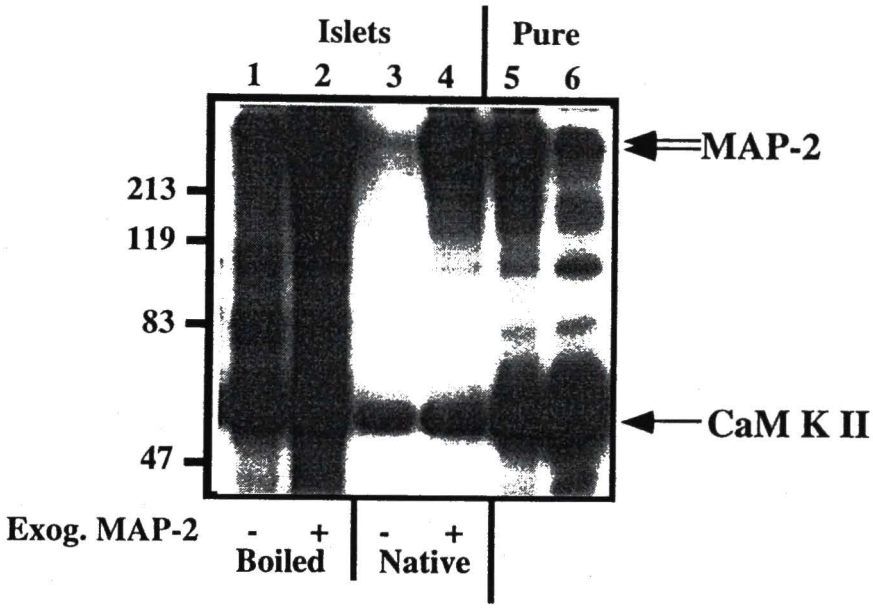
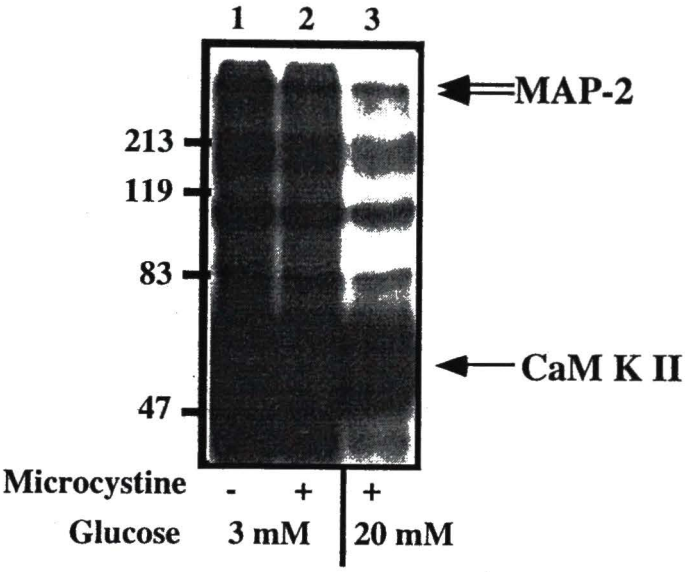
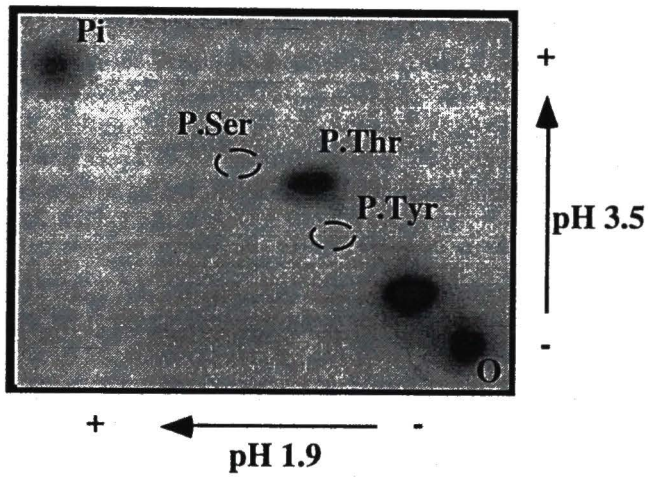

Islets
Pure
1 2 3 4 5 6
MAP-2
213
119
83
CaM K II
47
Exog. MAP-2
- + - +
Boiled
Native

FIG. 7. **Glucose stimulation attenuates MAP-2 phosphorylation by exogenous CaM kinase II in rat islets.** Isolated rat islets were stimulated by either KRB medium containing 3 mM glucose (*lanes 1* and *2*) or 20 mM glucose (*lane 3*). Samples were then subjected to back phosphorylation by the addition of CaM kinase II in the presence (*lanes 2* and *3*) or absence (*lane 1*) of microcystine (0.75 μM). MAP-2 was immunoprecipitated and phosphate incorporation determined by autoradiography.


1
2
3
MAP-2
213
119
83
CaM K II
47
Microcystine
-
+
+
Glucose
3 mM
20 mM

Phosphoamino Acid Analysis and Sequencing --- Phosphoamino acid analysis, an alternative approach to aid in the determination of the amino acid content of the CaM kinase II-specific sites on MAP-2 was also performed. Purified MAP-2 (10 μg) was phosphorylated by recombinant CaM kinase II as described, eluted from SDS polyacrylamide gels, and subjected to tryptic digestion (Krueger *et al.* 1997). After tryptic digestion, the resultant peptides were separated by electrophoresis in the horizontal dimension and by ascending chromatography in the vertical direction. The most prominent phosphopeptide was identified by autoradiography, scraped off the chromatography plate and subjected to amino acid hydrolysis in 6 *N* HCl. Separation of the individual phosphoamino acids of the hydrolysate was achieved by electrophoresis on cellulose thin-layer chromatography plates in two different dimensions. The comparison of the autoradiograph with the ninhydrin stained phosphoamino acid standards on the cellulose plate, unequivocably indicated that threonine was the radiolabeled amino acid in the peptide most prominently phosphorylated by CaM kinase II (Fig. 8). This phosphopeptide was also subjected to sequence analysis by mass spectroscopy but with inconclusive results, though another attempt in the future would be warranted.

FIG. 8. **Phosphoamino acid analysis of a MAP-2 peptide prominently phosphorylated by CaM kinase II *in vitro*.** Purified MAP-2 was phosphorylated by CaM kinase II and subjected to two-dimensional phosphopeptide mapping as described in Chapter III. The most prominently phosphorylated peptide was scraped off the cellulose plate, incubated in 6 *N* HCl at 110 °C for 1 hr, and the resulting hydrolysate further analyzed by electrophoresis in two dimensions with distinct buffers. The relative positions of the phosphoserine (P.Ser), phosphotyrosine (P.Tyr), orthophosphate (Pi), and the origin (O), are indicated. The spot closest to the origin likely represents partial hydrolysis products.


Pi
P.Ser
P.Thr
P.Tyr
O
+
pH 3.5
-
+
-
pH 1.9

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CHAPTER VI

FUTURE DIRECTION

Pancreatic β-cells contain a large number of insulin secretory granules of which only a small proportion are released even with maximal stimulatory conditions. Thus, the circulatory concentration of insulin is more dependent on the exocytotic process rather than the biosynthesis of the hormone itself. Regulation is exerted on the recruitment of secretory granules to the exocytotic site involving the cytoskeleton and its associated proteins, and on the targeting, docking and fusion of the secretory granules with the cell membrane. This entire process then is likely controlled by proteins localized on the secretory granule, in the cytosol and/or associated with the cytoskeleton, and on the plasma membrane. The determination of whether CaM kinase II is a key mediator in this process will most likely reveal its endogenous substrates in association with these cellular components.

This dissertation has evaluated the potential of two proteins, MAP-2 and synapsin I, to function as endogenous substrates for β-cell CaM kinase II. Continuing research examining the physiological significance of the phosphorylation of MAP-2 by CaM kinase II, has also been described. Interestingly, routinely co-immunoprecipitating with MAP-2, was another phosphoprotein of approximately 87 kDa (Fig. 4A, Chapter III). A previous study in which microtubule phosphopeptides were isolated also observed a 80 - 86 kDa phosphoprotein doublet which was later identified as synapsin I (Vallano *et al.* 1985). However, in the current study, an immunoblot analysis with an anti-synapsin I antibody failed to immunostain this mystery phosphoprotein. That this was not a function of the

polyclonal MAP-2 antibody was verified by the failure of the protein to be recognized by that same antibody in an immunoblot. The currently unidentified protein appeared to exhibit a time-dependent phosphorylation, though not a Ca^{2+}-dependent pattern like MAP-2. A possible candidate might be caldesmon, a 87 kDa protein and substrate for CaM kinase II. In smooth muscle, when phosphorylated, caldesmon is unable to bind to actin, and therefore is unable to block actin-myosin interaction. This results in activating smooth muscle contraction. Caldesmon is present in chromaffin cells and appears to be localized mainly to the cortical region of these cells (Trifaro *et al.* 1993). Possibly involved in the interplay between chromaffin granules and the cortical actin network, caldesmon while bound to actin under basal conditions, when activated by higher Ca^{2+} concentrations, dissociates from actin and interacts with chromaffin granules (Burgoyne *et al.* 1986). A testable hypothesis is that perhaps in the β-cell, caldesmon's association with actin evokes an inhibitory restraint on vesicle movement that is relieved upon phosphorylation of caldesmon by CaM kinase II.

The determination that synapsin I, while expressed in the islet, was not associated with insulin secretory granules, suggested an alternative association with another population of vesicles residing in the β-cell, synaptic-like microvesicles. Synaptic-like microvesicles of endocrine cells are similar to synaptic vesicles in membrane composition, biogenesis, and in the exoendocytotic recycling process. Studies have demonstrated that synaptic-like microvesicles in pancreatic β-cells store γ-aminobutyric acid (GABA), and that its release, like insulin, is differentially regulated by glucose (Thomas-Reetz *et al.* 1994; Gaskins *et al.* 1998). GABA has been demonstrated to modulate α-cell function in a negative paracrine manner (Gaskins *et al.* 1998; Bennett *et al.* 1983). Interest in β-cell GABA has increased following the identification of glutamic acid decarboxylase (GAD), a component of the pathway to GABA formation, as an early target antigen of the T-lymphocyte mediated destruction of the β-cell observed in IDDM (Atkinson *et al.* 1986).

Therefore, an extension of the current study of synapsin I would be to examine its potential association with synaptic-like microvesicles of the β-cell. This may be accomplished with the combined use of antibodies for synapsin I and synaptophysin, a known synaptic vesicle protein marker, in immunocytochemical techniques such as immunofluoresence and immunoelectron microscopy.

Interestingly, researchers, in initially determining whether the exocytosis of synaptic-like microvesicles was differentially regulated, noted that in neurons, differential release of the vesicles' cargo can be achieved by applying different frequencies of stimulation. A toxin from black widow spider venom, α-latrotoxin, was also demonstrated to induce massive exocytosis of acetylcholine from synaptic vesicles while leaving the large dense core vesicles seemingly unaffected (Trifaro *et al.* 1992). Moreover, in examining GABA secretion from pancreatic cells, AR42J cells, an amphicrine pancreatic cell line that expresses both exocrine and endocrine properties, exhibited differential release of GABA induced by elevated K^+ versus amylase release induced by the secretagogues bombesin and cholecystokinin (Thomas-Reetz *et al.* 1994). Differential regulation of the exocytosis of the two distinct vesicle populations in the β-cell may also occur, similar to that observed in the neuron. To that end, it might be noteworthy to repeat these investigations in the pancreatic β-cell. Specifically, incubation of isolated rat islets with α-latrotoxin followed by a perifusion collecting secreted insulin to quantify, may yield some interesting results. Concomitant analysis of CaM kinase II activity should also be performed.

Extensive research has been conducted investigating the regulation of neurotransmitter release by phosphorylation, specifically phosphorylation of synaptic vesicle-associated proteins. Future studies should take advantage of these data and test the possible application of such findings in the β-cell. Integral synaptic vesicle proteins have frequently been shown to exhibit potential phosphorylation sites. For example, synaptobrevin, synaptotagmin and synaptophysin, thought to play an important role in the

docking and/or fusion of synaptic vesicles with the presynaptic membrane have all demonstrated the ability to serve as substrates for CaM kinase II (Nielander *et al.* 1995; Popoli, 1993; Rubenstein *et al.* 1993). Synaptoporin, a homologue of synaptophysin, also thought to be involved in the formation of a fusion pore leading to synaptic vesicle exocytosis, contains two serine residues located within a consensus sequence for phosphorylation by CaM kinase II (Knaus *et al.* 1990). Rabphilin-3A, another synaptic vesicle protein, has proven to be an excellent substrate for CaM kinase II *in vitro* . Rabphilin-3A functionally interacts with rab3A, possibly mediating the fidelity of intracellular trafficking and membrane fusion events (Fykse *et al.* 1995).

Other possible sites of CaM kinase II action are cytoskeleton associated proteins, such as actin-associated proteins, α-actinin, gelsolin, scinderin, and fodrin, or the motor proteins, kinesin and the unconventional brain myosin V. α-Actinin, associated with chromaffin granules and the cell membrane, may function as an anchorage protein for the granules. Fodrin, a substrate for CaM kinase II, has been demonstrated in nerve terminals to cross-link synaptic vesicles to the plasma membrane via binding to synapsin I (Baines *et al.* 1985). Fodrin has also been found to mediate interactions between granules, actin, and the plasma membrane in chromaffin cells (Perrin *et al.* 1985). Gelsolin and scinderin activation result in the severing and depolymerization of actin filaments, with scinderin, in particular, demonstrating association with secretory events in adrenal cells (Vitale *et al.* 1991). Kinesin, a microtubule motor protein responsible for the intracellular transport of vesicles and organelles, has been characterized in the pancreatic β-cell (Balczon *et al.* 1992). Phosphorylation of neuronal kinesin was demonstrated to significantly reduce its binding to synaptic vesicles (Sato-Yoshitake *et al.* 1992). Moreover, antisense oligonucleotides to kinesin heavy chain resulted in the inhibition of insulin secretion from primary cultures of mouse β-cells (Meng *et al.* 1997). Brain myosin V is presently the leading candidate for transporting synaptic vesicles between their putative storage sites to their sites of release. It

has been demonstrated to form a stable complex with the synaptic vesicle membrane proteins, synaptobrevin and synaptophysin under basal conditions. With increased Ca^{2+} however, this complex was disrupted (Prekeris *et al.* 1997). Phosphorylation of brain myosin V by CaM kinase II has also been observed (Costa *et al.* 1996). These possible substrate candidates sharing common properties of cytoskeletal or vesicle associations and the potential to be phosphorylated, tempt future examinations as to their role as CaM kinase II substrates in the β-cell and possible involvement in the secretion of insulin.

An interesting observation of a large number of CaM kinase II substrates is the ability of these proteins to also be phosphorylated by other kinases. Noteworthy too, is the frequently shared characteristic of the electrostatic nature of binding between these substrates and other components, such that incorporation of a phosphate moiety can then functionally alter this type of binding interaction. The multifunctional property of CaM kinase II to phosphorylate a broad number of substrates is in apparent juxtaposition to achieving the observed specificity of targets. However, in reviewing the characterization of the synaptic vesicle-associated form of CaM kinase II (Benfenati *et al.* 1996), in which the kinetics of both autophosphorylation and substrate phosphorylation were indistinguishable from the soluble form of CaM kinase II, and yet, the selectivity of the interaction of synapsin I with the α-subunit of the kinase applied only to the synaptic vesicle-associated form of the kinase, it is obvious that an as yet undetermined factor conferring specificity is prominently functioning (i.e.; anchorage proteins or recalling the structure of CaM kinase II, the existence of unique heteroligomeric holoenzymes). That the interpretations of the mechanisms of exocytosis appear convoluted, reflecting the complexities of the process itself, are understandable and warranted. As stated by Schulman, "a hallmark of any signal transduction system is the presence of several levels of regulation..." (Schulman *et al.* 1989). Future research in comprehending the regulatory

role of CaM kinase II in insulin secretion will potentially improve the understanding of the mechanisms involved in insulin secretion and ultimately of the disorder, diabetes.

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