Perspectives in Diabetes
CaM Kinase II: A Protein Kinase With Extraordinary Talents Germane to Insulin Exocytosis

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CaM kinase II, a multifunctional Ca^{2+}/calmodulin-dependent protein kinase, is expressed in the pancreatic β-cell and is activated by glucose and other secretagogues in a manner correlating with insulin secretion. It is proposed that the activation of CaM kinase II mediates some of the actions of Ca^{2+} on the exocytosis of insulin secretory granules. This suggestion is supported by the localization of CaM kinase II to the insulin secretory granule and by the identification of two secretory-relevant proteins, MAP-2 and synapsin I, as endogenous substrates in the β-cell. Mechanistically, CaM kinase II appears to be involved in secretory steps proximal to granule fusion at the plasmalemma, and may facilitate protracted secretion through control of the interaction of granules with the cell cytoskeleton and their mobilization from intracellular synthesis sites. Through its unique regulatory properties, however, CaM kinase II is predicted to serve in more specialized aspects of the secretory process. In particular, the ability of CaM kinase II to remain active after cell stimulation is suggested to represent a mechanism by which releasable pools of granules are replenished between stimuli. *Diabetes* 48:675–684, 1999

The elevation of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in the pancreatic β-cell is central to the initiation of insulin secretion from the pancreatic β-cell under physiological conditions. All known physiological insulin secretagogues either induce the elevation of $[Ca^{2+}]_i$ or require that $[Ca^{2+}]_i$ be elevated to be effective. Glucose, for example, as the principal stimulus in man, is primarily dependent on the influx of Ca^{2+} from the extracellular medium, which is mediated by the indirect activation of voltage-dependent Ca^{2+} channels by signals derived from its metabolism (Fig. 1) (1,2). Ca^{2+} influx is intermittent, because of oscillatory changes in glucose metabolism, but is also strikingly synchronous with oscillatory insulin secretion (3,4). The fact that imposed oscillations of Ca^{2+} induce oscillatory secretion from permeabilized β-cells (5) strongly suggests that the former actually determines the latter. This appears to be the case in normal islets, where oscillations in insulin secretion occur only in the presence of Ca^{2+} oscillations (6), although arguments have also been forwarded that it is a metabolic signal that determines oscillatory insulin secretion (7). What is established, however, is that factors generated from the metabolism of glucose can enhance insulin secretion in the absence of changes in Ca^{2+}, although the precise nature of these factors has yet to be defined (Fig. 1) (8). Even in this case, a central role of Ca^{2+} is retained because these mechanisms still require that $[Ca^{2+}]_i$ be elevated (9). Recently, glucose has been shown to be capable of inducing insulin secretion in cells depleted of Ca^{2+} (10), but the contribution of these mechanisms to insulin secretion under physiological conditions is questionable (11).

Irrespective of whether Ca^{2+} drives or facilitates secretion, its central role demands that the specific proteins or intracellular mechanisms targeted by this cation be determined before insulin secretion is fully understood. From membrane capacitance measurements, it has been shown that changes in $[Ca^{2+}]_i$ localized to the plasma membrane region of the cell can elicit rapid insulin release through the direct modulation of fusion events (12). Studies from permeabilized β-cells, however, demonstrate that Ca^{2+}-dependent secretion is largely dependent on ATP and protein phosphorylation (13,14). From such information, it is hypothesized that insulin secretion is supported by the activation of Ca^{2+}-dependent protein kinases.

Ca^{2+}-DEPENDENT PROTEIN KINASES AND INSULIN SECRETION

Numerous Ca^{2+}-dependent protein kinases have been implicated in insulin secretion (Table 1) (15), but unequivocal evidence for functional involvement in insulin secretion is lacking. For example, Ca^{2+}-sensitive isoforms of the phospholipid-dependent protein kinase C (PKC) are translocated to membranes upon stimulation of the β-cell (16,17), but the physiological significance of this event is unclear. Rather, the inconsistent effects of enzyme downregulation on secretion induced by glucose leave conclusions about the role of PKC in this process controversial (16). In addition, mitogen-activated protein (MAP) kinase is a member of a distinct kinase cascade that is triggered in the β-cell in a manner dependent on Ca^{2+} influx (18,19). This kinase appears, how-

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$[Ca^{2+}]_i$, cytosolic Ca^{2+}; CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; MAP, mitogen-activated protein; MLCK, myosin light chain kinase; NSF, N-ethyl-maleimide–sensitive fusion protein; PKC, protein kinase C; PP-1, protein phosphatase-1; RT-PCR, reverse transcriptase–polymerase chain reaction; SLMV, synapsin-like microvesicles; α-SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.

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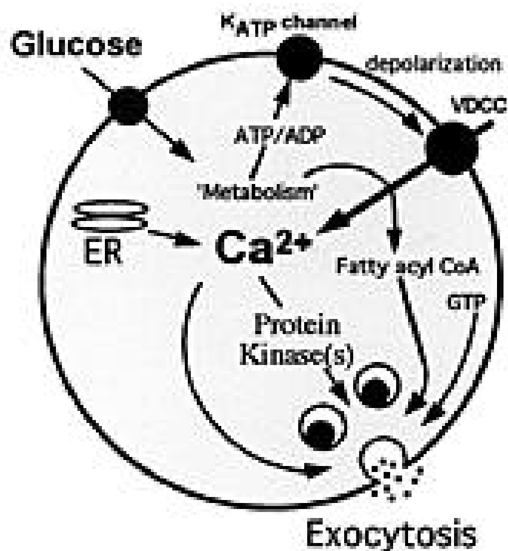

FIG. 1. Metabolic regulation of insulin secretion. Illustrated is a generalized scheme of glucose-induced insulin secretion from the pancreatic β-cell. The metabolism of glucose via glycolysis and oxidative metabolism in the mitochondria induces a rise in the cytosolic ATP/ADP, which, in sequence, closes K_{ATP} channels, induces membrane depolarization and opens L-type voltage-dependent Ca^{2+} channels (VDCC). Ca^{2+} induces insulin secretion in part by mechanisms that include the facilitation of granule fusion with the plasmalemma and the activation of protein kinases/phosphatases. Glucose-induced insulin secretion is also dependent on other factors derived from its metabolism, which may include adenine and guanine nucleotides (GTP) and the generation of long-chain fatty acyl CoAs.

ever, to have greater significance for the regulation of nuclear transcription events.

Since the discovery of calmodulin as a principal Ca^{2+} binding protein in the β-cell (25), the involvement of Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases) has been hypothesized as a primary mediator of the actions of Ca^{2+}. Accordingly, myosin light chain kinase (MLCK) activity was detected in islet and β-cell preparations over 15 years ago (21), fueling a suggestion that the phosphorylation of myosin light chain could effect secretion via the modulation of actin-myosin interactions (21,26). However, it is only recently that evidence has been gained for a role for MLCK in proximal granule mobility events (27,28). Around the same time, the β-cell was found to express an additional CaM kinase activity that, unlike the dedication of MLCK to a single substrate, displayed a multifunctional property to phosphorylate a diverse range of proteins (29,30). Later determined to be CaM kinase II, this kinase is activated by insulin secretagogues in a manner that correlates with insulin secretion (20), suggesting that it may play an important global regulatory role in the coordination of events relating to insulin exocytosis.

TABLE 1
Protein kinases or kinase cascades activated by Ca^{2+} in the β-cell

Kinase	Reference
CaM kinase II	20
Ca^{2+}/calmodulin-dependent MLCK	21
MAP kinase (ERK1/ERK2)	18,22
PKC	23
Phosphorylase kinase	24

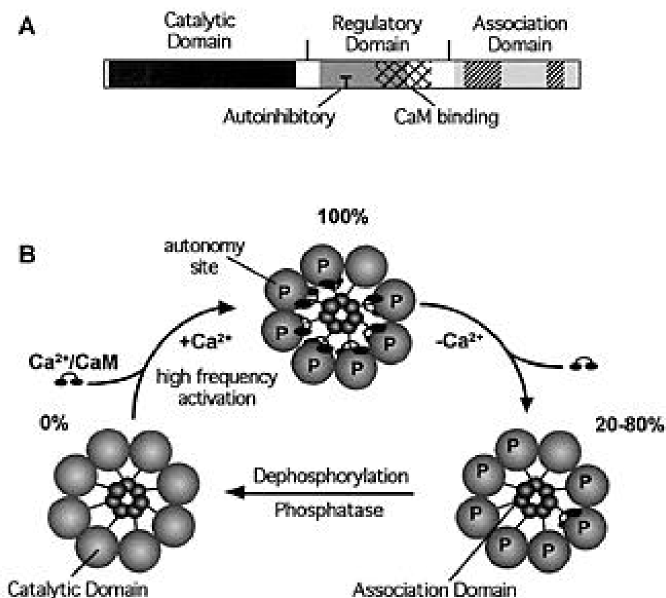

FIG. 2. Regulation of CaM kinase II. *A*: Domain structure of CaM kinase II. CaM kinase II isoforms are characterized by a highly conserved NH_2-terminal catalytic domain, a less highly conserved regulatory domain harboring overlapping autoinhibitory and calmodulin (CaM)-binding domains, and a variable COOH-terminal association domain. T indicates the threonine (Thr 286/287) residue that is autophosphorylated to generate an autonomous kinase. The hatched areas in the association domain indicate areas of diversity. *B*: Regulation of the holoenzyme by autophosphorylation. In vivo, CaM kinase II exists as a multimer of 8–12 subunits arranged in a hub and spoke structure that has been visualized by electron microscopy (36). In the presence of Ca^{2+}/calmodulin, individual subunits are activated, resulting in intermolecular autophosphorylation of neighboring subunits at Thr 286/287. Autophosphorylated subunits (P) have an increased (~1,000-fold) affinity for calmodulin, essentially trapping the cofactor within the multimer, so that in the event of a subsequent Ca^{2+} signal, the chances of autophosphorylation are increased. If frequent Ca^{2+} signals are detected, most subunits will be autophosphorylated (*top*). On the return of Ca^{2+} to resting levels, calmodulin eventually dissociates, while autophosphorylated subunits retain 20–80% activity in the absence of Ca^{2+}/calmodulin (autonomous activity). Under this condition, other sites may be autophosphorylated on CaM kinase II. Return to prestimulation levels requires the action of a phosphatase to remove the phosphate at the autonomy site.

EXPRESSION OF CAM KINASE II ISOFORMS IN THE PANCREATIC B-CELL

As repeatedly observed for other multifunctional kinases, molecular cloning has identified that CaM kinase II is a multigene family comprised of four distinct classes (α, β, γ, δ), each encoded by a separate gene (31). Classically, α and β CaM kinase II represent prominent neuronal isoforms, whereas γ and δ are more widely expressed in non-neuronal peripheral tissues (32). These isoforms display a high degree of structural identity (80–90%) in the NH_2-terminal catalytic and central regulatory domain (32) (Fig. 2*A*); the source of variation between classes is thus primarily accounted for by sequence differences in the COOH-terminal association domain (31). Within each class, 2–10 additional variants created from

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alternative splicing of messenger RNA transcripts have been cloned, giving rise to the 20 or so isoforms identified to date (31,33–35). This association domain is responsible for organizing 8–12 individual subunits into a multimeric hub and spoke complex necessary for its regulation by intersubunit autophosphorylation (36,37) (Fig. 2*B*). In addition, this domain is also thought to harbor targeting information such that sequence variations may determine the localization of different isoforms to distinct intracellular locations (31).

Attempts to identify the isoforms expressed in the pancreatic β-cell have resulted in numerous, but partially discordant, reports. For clarity, these are itemized in Table 2. The first β-cell isoform was cloned from neonatal rat islets and determined to be a novel β_3-isoform characterized by a proline-rich 86–amino acid insert in the association domain (35). This insert includes a putative SH_3 binding sequence, but speculation that this feature may target the kinase isoform to specific signaling components has yet to be substantiated. Nevertheless, the universal expression of CaM kinase II β_3 in human and rodent islets (35,38) and in clonal β-cell lines (39) hints that it may be of fundamental importance to β-cell function. In contrast, however, are reports of the differential expression of γ- and δ-isoforms in human versus rodent β-cell preparations. While δ_2, δ_6 (39), and δ_3 (R.A.E., M.J. Donelan, C.J. Rhodes, unpublished observations) have been reported as prominent isoforms in rodent β-cells, others have found no evidence for the expression of CaM kinase II-δ in human islets (38). These islets were found instead to express CaM kinase II-γ of standard subtypes γ_B and γ_E (38) and also a novel truncated CaM kinase II (γ_{SRP}) (40), which appears to be the product of a transpliced messenger RNA comprising the 5′ coding region of the kinase with the 3′ end of the human signal recognition particle SRP72. Takasawa et al. (41) have further reported the expression, though weak, of CaM kinase II-α in primary rat islet microsome fractions, but this observation cannot be reproduced in cultured β-cells (R.A.E., unpublished observations). The profile of CaM kinase II expression in the pancreatic β-cell within, as well across, species is far from settled. A cautionary note should also be added that many of these analyses are based on the amplification of total mRNA by reverse transcriptase–polymerase chain reaction (RT-PCR) and do not necessarily mean that an identified isoform is expressed to functional levels within the β-cell. Furthermore, since isolated islets are an aggregate of at least four distinct endocrine cell types (42), not to mention other non-endocrine cells that may be associated, RT-PCR products generated from primary tissue may not be representative of β-cell expression.

The clarification of this isoform profile is, however, important. It is likely that the β-cell simultaneously expresses more than one CaM kinase II isoform that, via their localization to specific areas of the cell, may regulate distinct cellular processes. By such a mechanism, multiple processes in the β-cell could be coordinately governed in response to an elevated $[Ca^{2+}]_i$ level, consistent with the proposed global regulatory role of CaM kinase II. This exciting possibility would be ideally probed using genetic approaches of kinase overexpression or knockout, but the intelligent application of these techniques can only be achieved with knowledge of specific isoform expression in the β-cell.

Two other points need to be mentioned here. First, the expression of CaM kinase II is not uniformly accepted, and a recent study using indirect immunofluorescence found little evidence for the expression of CaM kinase II in the β-cell (43). This study reported the alternative localization of CaM kinase II to the somatostatin-secreting δ-cell. Because the antibody used was raised against an epitope sequence conserved in all CaM kinase IIs, it is unlikely that this result reflects the recognition of an isoform not expressed in the β-cell. The discrepancy with other studies is, thus, not understood, but may be explained by an insufficient sensitivity to detect β-cell CaM kinase II or by the epitope being shielded in this cell. Second, CaM kinase II is just one of a family of calmodulin-dependent kinases that, in its broadest

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TABLE 2
Expression of CaM kinase II isoforms in β-cells

Isoform type	Isoform subtype	β-Cell preparation	Method of analysis	Reference
α	—	Rat islets	Western	41
β	β_3	Human islets Neonatal rat islets	RT-PCR	38
		HIT-T15 MIN-6	RT-PCR	35
		RINm5F	RT-PCR	39
γ	γ_B, γ_E	Human islets	cDNA screen/ RACE	38
	γ_{SRP}	Human islets	RACE	40
δ	δ_2	INS-1 cells RINm5F	RT-PCR Western	39
	δ_6 (originally δ_4)	RINm5F	RT-PCR	39
	δ_3	Rat islets Insulinoma	RT-PCR	*

*R.A.E., M.J. Donelan, C.J. Rhodes, unpublished observations. RACE, rapid amplification of cDNA ends.

sense, includes enzymes such as phosphorylase kinase and MLCK, as well as other closely related kinases, CaM kinases I, III, and IV (44). CaM kinase III is a dedicated elongation factor-2 kinase with the potential to regulate protein translation mechanisms, and evidence has been gained for its expression in rodent islets (45). CaM kinase I appears not to be expressed in the β- or δ-cell, but rather is localized to glucagon-secreting α-cells (43). There is no detectable expression of CaM kinase IV, at least based on immunological analyses, in the β-cell (A.R. Means, personal communication).

REGULATORY PROPERTIES OF CAM KINASE II

The more valuable insights into how CaM kinase II impacts cellular function have come from the elucidation of its regulatory properties. Akin to most other kinases, the catalytic site of inactive CaM kinase II is sterically occluded by interaction with an autoinhibitory sequence contained within the central regulatory domain, and is only relieved on activation by the binding of Ca^{2+}/calmodulin to an adjacent site (Fig. 2) (46,47). This opens the catalytic site to its substrates, facilitating the rapid intermolecular autophosphorylation of subunits contained within an oligomeric enzyme unit at a threonine residue in the autoinhibitory domain (Thr 286/Thr 287, depending on isoform) (48,49). Significantly, and unique to CaM kinase II, enzyme autophosphorylation changes the property of the enzyme in two major ways: *1*) autophosphorylation increases the affinity of the regulatory domain for calmodulin by ~1,000-fold. In effect, calmodulin becomes trapped on autophosphorylated subunits, thus increasing the chances of further activation under conditions of repetitive stimulation (50); *2*) Even when calmodulin finally dissociates, the autophosphorylated subunit retains partial activity (55% of full activity in β-cells [51]) in the absence of Ca^{2+}/calmodulin, termed "autonomous" activity (47). The enzyme is only inactivated by dephosphorylation of the autonomy site via the action of protein phosphatase(s). These properties bestow on the enzyme, at least in vitro, the ability to decode distinct frequencies of Ca^{2+} transients into appropriate levels of enzyme activation (52) and are central to its established role in models of synaptic plasticity and memory (53–55).

EVIDENCE FOR A ROLE OF CAM KINASE II IN INSULIN SECRETION

Enzyme activation. Quantitation of the extent of conversion of CaM kinase II to an autonomous form provided the first clear demonstration that glucose activates CaM kinase II in primary rat islets (20). Moreover, because enzyme activation mirrored insulin secretion, at least with respect to incremental increases in glucose concentration, these data provided significant support to the hypothesis that CaM kinase II is involved in the regulation of insulin secretion.

Several important aspects have emerged from subsequent studies. First, numerous other insulin secretagogues capable of elevating $[Ca^{2+}]_i$ concentrations have induced similar effects in clonal β-cells as well as islets (56,57). Thus, enzyme activation is surely important to the β-cell, and cannot be dismissed as a regulatory process confined to mantle cells of the islet. Second, in permeabilized clonal βTC3 cells, similar concentrations of Ca^{2+} are required to induce CaM kinase II activation and insulin secretion (51,58) consistent with a functional relationship of the two parameters. Moreover, the desensitization of secretion characteristic of these cell preparations correlates with a loss of CaM kinase II activity (59). A third point worth noting is that CaM kinase II activation is universally achieved by insulin secretagogues that promote Ca^{2+} influx from the extracellular medium (e.g., glucose, K^+) (20,56), as well as Ca^{2+} mobilization from intracellular stores (e.g., the acetylcholine analogue, carbachol [57], and cholecystokinin [R.A.E., unpublished observations]), although the temporal profiles of activation seem distinct. This may prove important because individual β-cell events may be controlled by specific Ca^{2+} pools. Finally, and perhaps most significantly, the activation of CaM kinase II correlates temporally with insulin secretion from perifused rat islets (60). This relationship is reproduced in Fig. 3. Under these conditions, CaM kinase II activation occurs simultaneously with the initiation of insulin secretion, but is also sustained throughout the second phase of hormone release. Activation levels achieved during this latter stage were virtually maximal (based on criterion described by Easom et al. [51]), suggesting the complete conversion of the enzyme to the autonomous form. This temporal correlation provides the most compelling evidence to date that CaM kinase II plays an important role in the regulation of glucose-induced insulin secretion.

Enzyme inhibition. A rather different and confused story is derived from inhibitor studies. Initially, a positive role of CaM kinase II was supported by observations that isoquinoline sulfonamide derivatives with putative selectivity toward CaM kinase II, KN-62, and KN-93 (61,62), suppressed insulin secretion from isolated islets (63,64) or cultured β-cells (HIT-T15) (65). However, it is now clear that these effects are primarily attributable to the inhibition of L-type Ca^{2+} channels rather than direct effects on the kinase (65,66). In fact, from an additional observation that KN-62, failed to suppress Ca^{2+}-induced insulin secretion from permeabilized β-cells, conditions that are deemed independent of Ca^{2+}-channel activity, investigators were led to conclude the opposite, that CaM kinase II plays no functional role in insulin secretion (65).

This latter conclusion seems inconsistent with activation data, and it contradicts other reported effects of the peptide

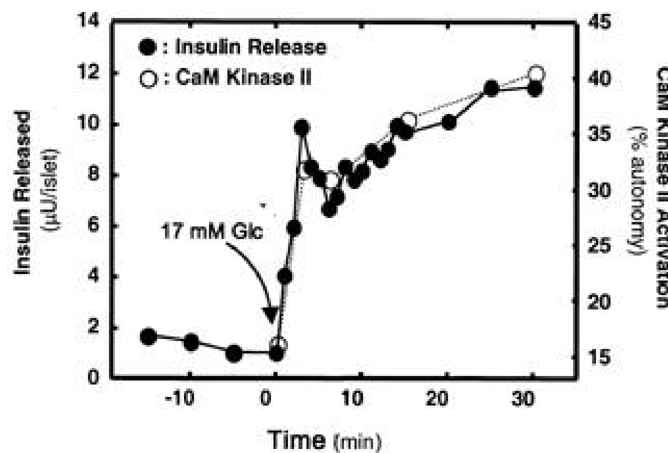

FIG. 3. CaM kinase II activation correlates with insulin secretion from perifused rat islets. Isolated rat islets were stimulated with glucose at time 0 (indicated by the arrow), and insulin secretion and CaM kinase II activation were determined from the same islets at the indicated times. CaM kinase II activation is given as a ratio of autonomous to CaM kinase activities, and is a measure of the extent to which the enzyme is converted to an autonomous form. Adapted from Easom et al. (60).

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inhibitor CaMK 290-309 to significantly reduce cell depolarization-induced β-cell exocytosis as determined by patched-cell capacitance measurements (67). Upon reexamination, experiments in my laboratory suggest that the inability of KN-62 to influence Ca^{2+}-induced insulin secretion from permeabilized β-cells can be explained by its inability to inhibit CaM kinase II activity in these preparations (H. Bhatt, R.A.E., unpublished observations). The reason for this is not understood, but under the same experimental conditions, insulin secretion is markedly suppressed by KN-93 and the peptide inhibitors of CaM kinase II, AIP (68) and [Ala^{286}]-CaMK (281–302) (69) (H. Bhatt, R.A.E., unpublished observations). Thus, these latest inhibitor studies do support a role of CaM kinase II in insulin secretion, but they also highlight significant specificity and interpretative problems surrounding the use of current inhibitors of CaM kinase II. There remains no reliable method to pharmacologically suppress CaM kinase II in the intact β-cell to permit an assessment of its role in glucose-induced insulin secretion, so alternative experimental strategies have been developed.

Intracellular localization of CaM kinase II to insulin secretory granules. The targeting principles of signal transduction reason that the specificity of the action of a multifunctional kinase such as CaM kinase II is determined to some degree by its intracellular location (70). It is therefore of significant interest that two independent studies have reported the specific association of CaM kinase II with purified fractions of insulin secretory granules obtained from insulinoma tissue or β-cell lines (39) (R.A.E., M.J. Donelan, C.J. Rhodes, unpublished observations). Although both studies suggest that this granule-associated kinase is of a δ isoform class, there is again some dispute as to its identity as either δ_2 or δ_3. What is more critical with respect to function, however, is that this granule-associated CaM kinase II is shown to be maximally activated (autophosphorylated) by Ca^{2+} within a concentration range required to support insulin secretion (51). The very positioning of CaM kinase II on the insulin granule strongly implicates it in the secretory process and, as such, is analogous to the neuron where CaM kinase II is a prominent surface protein of the synaptic vesicle (71).

Identification of intracellular substrates of CaM kinase II in the β-cell. An understanding of the relevance of CaM kinase II activation to insulin secretion, or other β-cell functions, is dependent on the identification of proteins phosphorylated by this kinase and the effect these modifications have on their function. An impressive list of proteins are known to be phosphorylated by CaM kinase II in vitro (31), but few have been proven to be substrates in vivo. Among this latter group, however, are the neuronal proteins synapsin I (72) and MAP-2 (73), both of which are thought to function in neuronal secretion. Many aspects of neuroexocytosis appear to be conserved within the β-cell. It is, therefore, significant that recent studies have established that both MAP-2 (58) and synapsin I (74,75) are expressed in primary islet β-cells and that each serves as an endogenous substrate for CaM kinase II under conditions that support insulin secretion (51,58,74). To date, these are the only validated substrates of CaM kinase II in the β-cell.

CAM KINASE II-MEDIATED PHOSPHORYLATION AND INSULIN SECRETION

What role is CaM kinase II anticipated to play in insulin secretion? The correlation of enzyme activation and insulin secretion has been used as primary evidence for their functional relationship, but these parameters can be dissociated under certain conditions. For example, glucose-induced activation of CaM kinase II in perifused islets is not affected by addition of inhibitory hormones, somatostatin or epinephrine, which cause profound suppression of insulin release (60). Furthermore, a maximal activation state of CaM kinase II is transiently maintained (for ~5 min) after the removal of the glucose stimulus, despite a rapid termination of insulin release (60). The implications of these observations are best discussed within the context of the putative mechanism of insulin secretion (Fig. 4).

Granule fusion. The final steps of insulin exocytosis are mediated by a sequence of kinetically distinguishable steps (termed the SNAP-receptor [SNARE] hypothesis) whereby granules dock, undergo priming, and then fuse with the plasma membrane. The initial interaction is mediated by cognate proteins of the vesicle (*v*-SNAREs) and the plasmalemma (*t*-SNAREs) that recognize each other in a lock and key fashion to form a multisubunit complex (Fig. 4*B*) (76). This docking complex then becomes a receptor for NSF (N-ethylmaleimide–sensitive fusion protein) and α-SNAP (soluble NSF attachment protein), the former having an ATPase activity that furnishes the energy required to create fusion-competent granules (termed "priming") (77). There is consensus within both the β-cell (12) and other neuroendocrine cells (e.g., chromaffin) (77–79) that this priming mechanism is the last ATP-dependent step of exocytosis. Granule fusion, per se, thus occurs independent of protein kinase involvement and is thought to be mediated by the direct action of Ca^{2+} through Ca^{2+}/phospholipid binding proteins, synaptotagmin (80) or syncollin (81). Micromolar concentrations of Ca^{2+} are required to support granule fusion, and this is likely achieved by localization of L-type Ca^{2+} channels to the exocytotically active regions of the cell (82) (Fig. 4*B*). The predicted irrelevance of CaM kinase II activation to granule fusion is consistent with its insensitivity to somatostatin and epinephrine because the inhibition of insulin secretion by these hormones is mediated by G-proteins (G_i, G_o) acting at some postpriming step (83,84).

Granule docking and priming. Only a minimal fraction (estimated at <1%) of the β-cell granules are in a primed, readily releasable state (12,85), although the release of this pool appears sufficient to account for the first phase of insulin secretion (12). Protracted secretion is, therefore, dependent on the recruitment of granules from additional, or reserve, pools. In fact, based on the smaller size of this readily releasable pool of granules in the β-cell relative to other endocrine cells (i.e., 40–140 vs. 1,000–3,000 estimated for pituitary melanotrophs or adrenal chromaffin cells [12,79]), recruitment represents a more important component of insulin exocytosis. Granule recruitment, within this context, encompasses granule docking and the mobilization of granule pools restrained at intracellular areas removed from the plasma membrane and represents a more likely site for the intervention of CaM kinase II.

MECHANISMS OF GRANULE RECRUITMENT AS A TARGET OF CAM KINASE II ACTIVATION

SNARES. The β-cell expresses vesicle-associated membrane protein (VAMP-2)/synaptobrevin and cellubrevin as *v*-SNARES and SNAP-25 and syntaxin as *t*-SNAREs (86).

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Their importance to exocytosis is established from demonstrations that clostridial toxins that specifically target individual SNARE proteins inhibit insulin secretion (76). How this process is regulated is still a complex question, but the involvement of CaM kinase II is implicated by its ability to phosphorylate a number of SNARE proteins, i.e., VAMP (or synaptobrevin), NSF, syntaxin, SNAP-25, α-SNAP, and synaptotagmin, in vitro (72,87,88). This is, however, only speculation, since it has yet to be determined whether any of these factors serve as substrates for CaM kinase II in vivo in any tissue.

Synapsin I. The phosphorylation of synapsin I by CaM kinase II has been proposed as a key mechanism in the recruitment of synaptic vesicles for release. Because many features of neuroexocytosis are conserved in the β-cell, it was reasoned that this prominent CaM kinase II substrate might serve a similar role in the β-cell. The classical view that synapsin I is not expressed in the β-cell (89) was recently challenged, first by the report of a synapsin-like protein (molecular weight 84,000) in the β-cell line (MIN-6) (75), and then by the demonstrated expression of synapsin I in βTC3 cells and

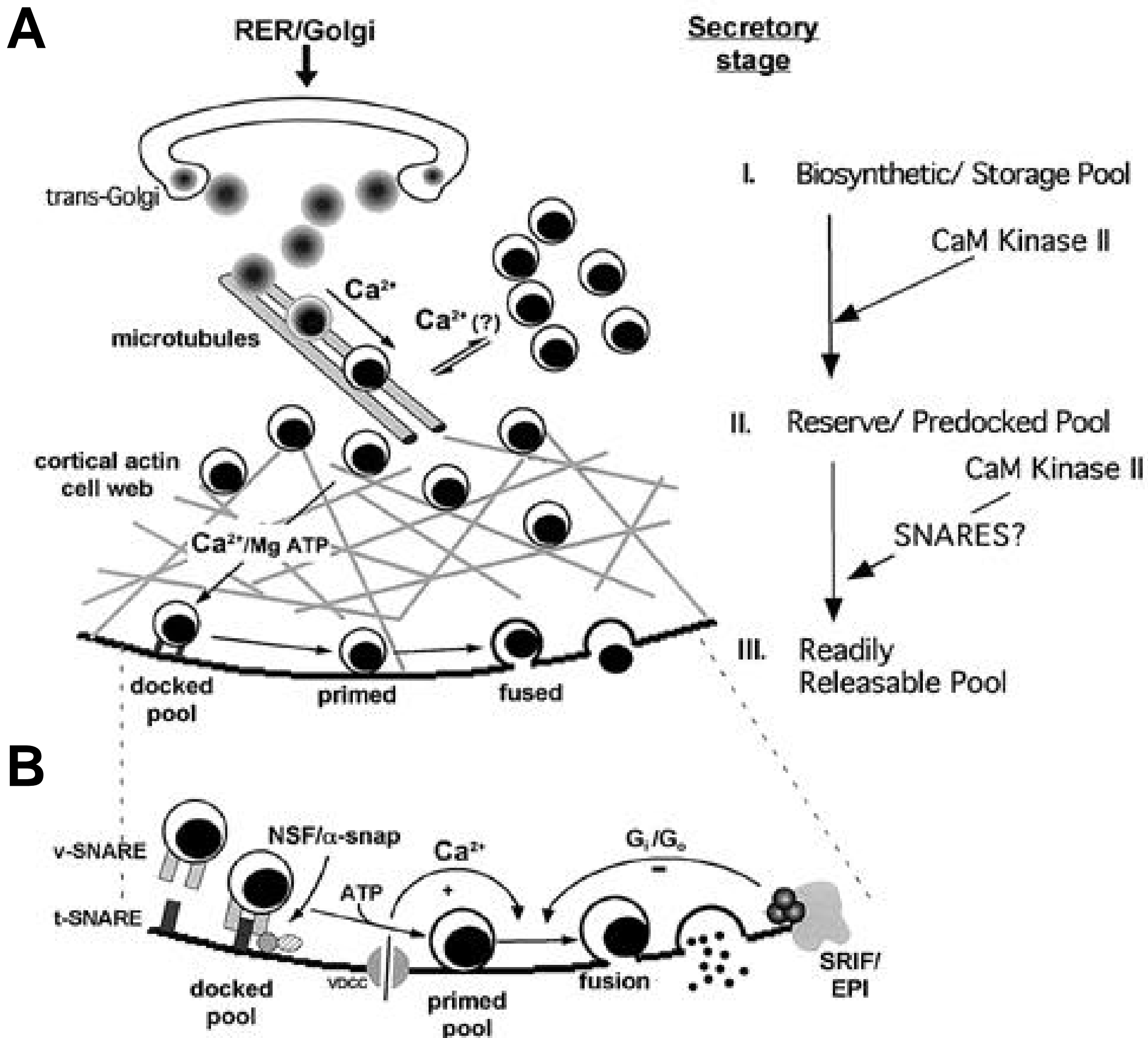

FIG. 4. Mechanism of insulin exocytosis: potential modulation by CaM kinase II. *A*: Insulin secretion involves the synthesis and packaging of proinsulin in immature granules at the trans-Golgi, the subsequent translocation of processed granules through the cortical cell web, and the fusion of granules with the plasma membrane to release mature insulin. This process has been broken down into stages representing distinct pools of granules. *Stage I*: Biosynthetic pool. Granules released from the trans-Golgi interact with the microtubules and are transported toward the cell periphery, possibly through the MAP-2 phosphorylation by CaM kinase II (reserve pool). *Stage II*: Granules restrained by the cell web are released to dock with the plasma membrane. CaM kinase II is potentially involved via the regulation of microtubule-microfilament interactions (MAP-2) or through the phosphorylation of SNARE proteins in docking or priming mechanisms. *Stage III*: Granules primed at the plasma membrane are triggered to fuse with the plasmalemma to release their contents to the extracellular medium. This stage is thought not to involve CaM kinase II. *B*: Enhanced view of exocytosis events at the plasma membrane indicating the involvement of SNARES, the cellular localization of L-type Ca^{2+} channels, and the interaction of heterotrimeric G-proteins activated by inhibitory hormones, somatostatin (SRIF), or epinephrine (EPI).

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primary rat islets (74). As in the neuron, synapsin I is phosphorylated at CaM kinase II–specific sites in the β-cell when the intracellular Ca^{2+} is elevated to a level that supports insulin secretion (51,74). By immunofluorescence, synapsin I and insulin are similarly distributed in the β-cell (R.A.E., K.A. Krueger, unpublished observations), but more detailed studies using immunogold electron microscopy do not support a colocalization of synapsin I with insulin granules (74). Because no evidence has been gained for the phosphorylation of synapsin I on isolated insulin granules, this information collectively argues against a role in the exocytosis of these granules. Rather, the grouping of immunolabeled synapsin I to smaller vesicle-like structures in β-cell preparations suggests that synapsin I may be important for the trafficking of other non–insulin-containing vesicles (74). Of potential interest here is that the β-cell expresses synaptic-like microvesicles (SLMV) that package the neurotransmitter, GABA (90), and are secreted in a glucose-dependent manner (91). It is not clear yet whether synapsin I is specifically localized to these vesicles, but the exciting possibility that CaM kinase II regulates SLMV exocytosis is confused by the reported absence of CaM kinase II on preparations of these vesicles (39).

The consequence of synapsin I phosphorylation by CaM kinase II to β-cell function is obviously not understood. A hindrance here is that the function of synapsin I in neurons, where it is expressed to much higher levels, remains unclear. Recent data from synapsin I and synapsin I/II knockout mice actually argue against an essential role of synapsin I in neurotransmitter release (92,93). The most evident defects in these animals are in short-term synaptic plasticity, suggesting a more specialized role of synapsin to accelerate vesicle cycling under conditions of repetitive nerve stimulation. The keys to its function in both neurons and β-cells may well lie in recent evidence, from the delineation of its crystal structure, that synapsin I possesses ATPase activity and may also function as a phosphoryltransferase (94,95).

MAP-2. The phosphorylation of MAP-2 by CaM kinase II is likely to be significant because of the assumed role of microtubules in insulin exocytosis, shown principally from studies using mitotic spindle inhibitors (96,97). These drugs inhibit insulin secretion, irrespective of whether their end result is the disruption of microtubules, e.g., colchicine and nocodazole, or their stabilization, e.g., D_2O and taxol, implying that it is the dynamic process of microtubule assembly/disassembly that is important to the secretory process. Microtubule stability is, in turn, controlled by the phosphorylation state of microtubule-associated proteins (98). In the case of MAP-2, its phosphorylation by CaM kinase II, at least in vitro, reduces its interaction for tubulin favoring microtubule breakdown (73).

MAPs appear to be important in secretory processes because *1*) isolated insulin secretory granules interact with microtubules only in the presence of exogenous MAPs (99); *2*) MAPs form thin bridges between granules and microtubules (100); and *3*) estramustine phosphate, a drug that selectively interacts with the microtubule binding domains of MAPs, inhibits insulin secretion (R.A.E., B. Conner, unpublished observations). Little to no MAP-2 is detectable on purified preparations of isolated insulin secretory granules, but it can be phosphorylated by CaM kinase II when added exogenously to these preparations (R.A.E., M.J. Donelan, C.J. Rhodes, unpublished observations). It is possible, therefore, that the juxtaposition of granule-associated kinase and cytoskeletal MAP-2 regulates the interaction of granules with the microtubule network on which they can be transported under the power of motor proteins such as kinesin. This mechanism is, again, suggested to be involved in granule recruitment because both estramustine phosphate (R.A.E., B. Conner, unpublished observations) and microtubule inhibitors (96) selectively suppress second-phase secretion. Other studies, using video microscopy, have provided evidence for a positive role for CaM kinases in insulin granule migration (101), but the extent to which this reflects the modulation of microtubule dynamics is yet to be determined .

The phosphorylation of MAP-2 may have a broader role in insulin secretion, as suggested by its involvement in the interaction of microtubules with actin microfilaments (102,103). In the β-cell, microfilaments form a cell web that appears to act as a barrier to granule migration to the plasma membrane, although it may also provide motility force through its contraction under cell stimulation (104). The rearrangement of this cortical actin web in neuroendocrine cells is Ca^{2+}-dependent (105), and a possible role of CaM kinase II is implicated by its localization to the terminal cytoskeletal web region of ileal enterocytes (106).

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SIGNIFICANCE OF CAM KINASE II AUTOPHOSPHORYLATION TO INSULIN EXOCYTOSIS

It is expected that the unique regulatory potential of autophosphorylated CaM kinase II would be a prominent reason for its use in any cellular function. This principle is exemplified by the neuron, where these properties equip the enzyme with the ability to function as a molecular sensor of synaptic usage important in the cellular paradigms of learning and memory (55). What is the significance of CaM kinase II autophosphorylation to the pancreatic β-cell? Because of the potential of CaM kinase II to decode frequencies of intracellular Ca^{2+} oscillations, it is possible that β-cell isoforms function to direct the appropriate secretory response to oscillatory changes induced in $[Ca^{2+}]_i$ by various combinations of secre-

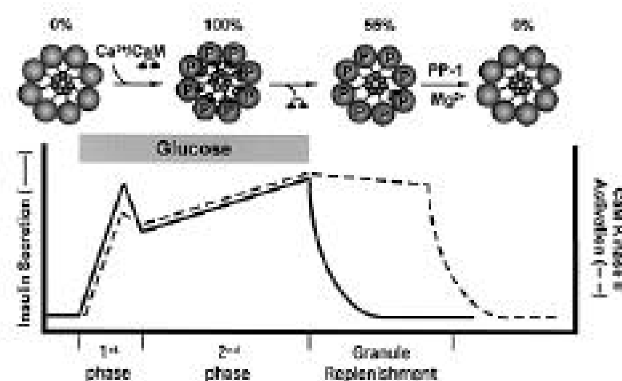

FIG. 5. CaM kinase II autophosphorylation in the mechanism of granule replenishment. On the withdrawal of glucose, CaM kinase II activation (- - -) is maintained for ~5 min, while insulin secretion is rapidly quenched (60). Enzyme activation in the presence of Ca^{2+}/calmodulin is maximal during second-phase secretion. After glucose withdrawal, calmodulin (CaM) dissociates, revealing the autonomous kinase that is calculated to have ~55% of the fully active enzyme (51). The deactivation of the enzyme must correlate with dephosphorylation of the autonomy site of CaM kinase II, which, in the β-cell, involves PP-1 plus an additional Mg^{2+} factor (51). The retention of kinase activity is thought to permit the replenishment of releasable granule pools.

tagogues. On a simpler level, the inherent ability of autophosphorylated CaM kinase II to maintain substrate phosphorylation based on a past stimulus may be useful, and even critical, to the functional capacity of the β-cell. Presumably, the ability of the cell to respond appropriately to repeated stimuli requires that the relevant pools of granules be adequately stocked. As a potential motivator of granule recruitment, the activation of CaM kinase II may be important not only for the support of second-phase secretion during the period of the stimulus, but also for the replenishment of these granule pools after the stimulation has ceased. In the latter case, the retention of autonomous kinase activity after the removal of glucose may be necessary to ensure continued granule recruitment (Fig. 5). Within this context, the demonstration that autonomous kinase activated on secretory granules can phosphorylate MAP-2 in a Ca^{2+}-independent manner is important, and implies that poststimulation control of microtubule function by CaM kinase II is feasible (R.A.E., M.J. Donelan, C.J. Rhodes, unpublished observations).

REGULATION OF CAM KINASE II PHOSPHATASE

Given that CaM kinase II autophosphorylation is at the heart of its contribution to β-cell function, the control of the protein phosphatase that dephosphorylates the autonomy site and returns the enzyme to an inactive state becomes very important. The period during which autonomous CaM kinase II activity can influence cellular processes is presumably determined by the activity of phosphatases. Within the model proposed for granule recruitment, the degree to which granule pools are replenished might be dependent on the ability of the cell to restrain CaM kinase II phosphatase activity (Fig. 5). For this reason, effort has been dedicated to the identification of this protein phosphatase in the β-cell. By traditional techniques that rely on established sensitivities of prominent protein phosphatases to various inhibitors, a protein phosphatase-1 (PP-1)-like activity was found to be involved in the deactivation of CaM kinase II in β-cells in situ (51). This study, however, also indicated that PP-1 is maximally effective only in synergy with a further Mg^{2+}-dependent factor, possibly itself a phosphatase. It is perhaps not surprising to find that this mechanism, with its potential to profoundly influence β-cell function, is multifaceted and complex. Its elucidation is thus anticipated to hold significant clues to the intricate workings of CaM kinase II in the β-cell.

CLOSING REMARKS

Is CaM kinase II the Ca^{2+}-sensitive protein kinase regulator of insulin exocytosis that has been anticipated for so long? The preliminary observations clearly warrant future interest. Its partial localization to the insulin secretory granules places it in perfect position to oversee secretion, and its regulatory prowess seems appropriate to drive the secretory demands of the cell both during the stimulation period and perhaps at later time points in preparation for the next stimulus. It is not implied, however, that CaM kinase II works alone. Glucose can enhance insulin secretion in the absence of changes in $[Ca^{2+}]_i$ (9), or in its absence altogether (107), although such processes could be supported by lingering autonomous kinase activity. In addition, it has not escaped attention that the intracellular β-cell proteins known to be targeted by CaM kinase II (i.e., MAP-2 and synapsin I) are also substrates for protein kinases A and C. Both kinases are capable of enhancing the responsiveness of the distal steps of the secretory process to Ca^{2+} (15,108,109). Thus, with CaM kinase II as a possible major conduit for the influence of Ca^{2+} on insulin secretion, it would seem appropriate that its substrates would serve as recipients of such synergy. As more substrates are identified for CaM kinase II in the β-cell, it is anticipated that an important central role for this kinase in insulin exocytosis will become increasingly apparent.

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