Calcium-stimulated Phosphorylation of MAP-2 in Pancreatic βTC3-cells Is Mediated by Ca\textsuperscript{2+}/Calmodulin-dependent Kinase II*

(Received for publication, March 11, 1997, and in revised form, August 12, 1997)

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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\textsuperscript{2+} or addition of forskolin, an adenylate cyclase activator, induced significant phosphorylation of MAP-2 in situ. The effect of Ca\textsuperscript{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\textsuperscript{2+}. Phosphopeptide mapping revealed that the phosphorylation pattern observed in situ upon incubation of the βTC3 cells with increased free Ca\textsuperscript{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\textsuperscript{2+}-dependent insulin secretion.

Circumstantial evidence supports a functional role of the multifunctional Ca\textsuperscript{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 (1), activates CaM kinase II in isolated rat islets in a concentration-dependent manner (2) that temporally correlates with the initial and sustained phases of insulin secretion (3). 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 (4, 5), although conclusions made from such studies are complicated by nonspecific effects demonstrated by these drugs (6, 7). Another study that reports the inability of KN-62 to inhibit Ca\textsuperscript{2+}-induced insulin secretion from the permeabilized β-cell (7) 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 (8), but relatively few of these 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 (9) or hippocampal slices (10) 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 (11) as well as their interaction with other cellular structures such as actin (12). This regulatory capacity is in turn controlled by the phosphorylation state of MAP-2, at least in vitro (13). Although a minimal extent of MAP-2 phosphorylation appears to be essential for MAP-2 function (14), 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 (15). In optimal conditions, isolated MAP-2 has been demonstrated to incorporate phosphate to the level of 46 mol/mol of MAP-2 (16). Although MAP-2 is phosphorylated by multiple protein kinases including the phospholipid-dependent protein kinase C (17) and the AMP-dependent protein kinase (PKA) (18), 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 (19).

Based on the established involvement of the microtubule network in insulin secretion (20–23) and the suspected association of CaM kinase II with the cytoskeleton of the β-cell (24), 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\textsuperscript{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

* This work was supported by Grant DK47925 from the National Institutes of Health (to R. A. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: CaM kinase II, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II; MAP-2, microtubule-associated protein-2; PKA, protein kinase A; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone.
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. Protein A-Sepharose, monoclonal anti-MAP-2 (clone Einstein College of Medicine, New York). RPMI 1640, glutamine, antibiotics, trypsin/EDTA, and fetal bovine serum were purchased from Life Technologies, Inc. Protein A-Sepharose, monoclonal anti-MAP-2 (clone Einstein College of Medicine, New York). RPMI 1640, glutamine, antibiotics, trypsin/EDTA, and fetal bovine serum were purchased from Life Technologies, Inc. Protein A-Sepharose, monoclonal anti-MAP-2 (clone Einstein College of Medicine, New York). RPMI 1640, glutamine, antibiotics, trypsin/EDTA, and fetal bovine serum were purchased from Life Technologies, Inc. Protein A-Sepharose, monoclonal anti-MAP-2 (clone Einstein College of Medicine, New York). RPMI 1640, glutamine, antibiotics, trypsin/EDTA, and fetal bovine serum were purchased from Life Technologies, Inc. Protein A-Sepharose, monoclonal anti-MAP-2 (clone Einstein College of Medicine, New York). RPMI 1640, glutamine, antibiotics, trypsin/EDTA, and fetal bovine serum were purchased from Life Technologies, Inc. Protein A-Sepharose, monoclonal anti-MAP-2 (clone Einstein College of Medicine, New York). RPMI 1640, glutamine, antibiotics, trypsin/EDTA, and fetal bovine serum were purchased from Life Technologies, Inc. Protein A-Sepharose, monoclonal anti-MAP-2 (clone Einstein College of Medicine, New York).

**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% CO₂. In preparation for permeabilization, βTC3 cells were detached (Trypsin/EDTA) and equilibrated in suspension culture medium for a minimum of 2 h. Following a brief centrifugation, the cells were washed twice with Ca²⁺/Mg²⁺-free Krebs-Ringer bicarbonate/Hepes buffer (25 mM Hepes, pH 7.4, 115 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, and 1 mM MgCl₂) containing 1 mM EGTA, 6 µM glucose, and 0.1% bovine serum albumin. After counting, permeabilization was initiated by the addition of S. aureus toxin, α-hemolysin, to a concentration of 125–200 units/10⁶ cells/0.1 ml Ca²⁺/Mg²⁺-free permeabilization buffer (20 mM Hepes, pH 7.9, 140 mM potassium glutamate, 5 mM NaCl, 4 mM MgSO₄, 1 mM EDTA, and 300 µM Na ATP). Permeabilization was conducted at 37 °C for 15 min with the efficiency monitored by visualizing trypan blue accessibility to the intracellular space without the loss of the intracellular protein (28).

**Isolation of Pancreatic Islets**—Pancreatic islets were isolated from male Wistar rats (Harlan Sprague-Dawley, Indianapolis, IN) by collagenase P (Boehringer Mannheim) digestion and subsequent enrichment by centrifugation on a Ficoll gradient as described previously (2). Immuno blot 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 activity, 5 × 10⁵ permeabilized cells were incubated in buffer (500 µl) containing varying concentrations of free Ca²⁺ 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 (29). ³²P-incorporation into autocamtide-2 was determined by Cerenkov radiation (Beckman). The activity of CaM kinase II in the absence of Ca²⁺/calmodulin (autonomous activity) expressed as percentage of total activity in the presence of Ca²⁺ was used as a measure of enzyme activation.

**MAP-2 Phosphorylation and Immunoprecipitation in Situ**—Immunoprecipitation conditions were optimized for specific activity of ³²P-labeled MAP-2 phosphorylated by PKA catalytic subunit, or mouse recombinant CaM kinase IIα as described (9) with the following exceptions; the PKA mixture was without exogenously added CaCl₂, and the reaction volume of 50 µl contained [³²P]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, ³²P-labeled MAP-2 was eluted from gel slices by incubation in 50 mM NH₄HCO₃, pH 7.3–7.8, initially supplemented with 0.1% mercaptoethanol and 0.01% SDS for 1 h and then without supplement for a further 3 h. The eluates were pooled, and the eluted MAP-2 was precipitated by the addition of a final concentration of 16% trichloroacetic acid (for 1 h on ice) in the presence of 20 µg heat-denatured RNase as carrier. In situ phosphorylated MAP-2 was similarly precipitated at this step. The precipitate was washed sequentially in oxidizing solution (50 µl of performic acid) and then digested by the addition of 10 µg of trypsin for 1 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 HITE 7000 thin-layer electrophoresis apparatus (C. B. S. Scientific, La Jolla, CA) as described (30 except that the electrophoresis and chromatography steps were conducted at 1.3 kV for 25 min and for 14 h using a phospho chromatography 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 three 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 Dunnett's 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 (31–33) accounting for as much as 1% of the total cytoplasmic protein. In contrast, MAP-2 levels are much lower in non-neuronal tissues (34) but demonstrated to be expressed in secretory cells, rat glioma (35), pituitary and PC12 (36). By immunoblot analysis using a polyclonal anti-MAP-2 antibody, βTC3 cells were demonstrated to express a high molecular weight protein (M₉ > 205 kDa) of electrophoretic mobility indistinguishable from MAP-2 purified from bovine brain (Fig. 1A, lane 1 versus lane 4). This MAP-2-like protein was immunoprecipitated from βTC3 cell homogenates by this antibody as indicated by its disappearance from β-cell homo-
Ca\(^{2+}\) (to mimic the intracellular concentration of a resting β-cell (41)) \(^{32}\)P was incorporated into MAP-2 in a time-dependent manner (Fig. 2). 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 (14). On elevation of the Ca\(^{2+}\) concentration to 5 μM (to promote the activation of CaM kinase II) the extent of \(^{32}\)P incorporation into MAP-2 was significantly increased; at the optimal time of 1 min, 5 μM Ca\(^{2+}\) increased \(^{32}\)P incorporation into MAP-2 by 326 ± 76% relative to time 0, 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).

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.
profiles is consistent with a functional association of Ca\(^{2+}\)-dependent activation of CaM kinase II with the phosphorylation of \(\beta\)-cell MAP-2 and is further substantiated by virtually identical Ca\(^{2+}\)-dependence of CaM kinase-mediated phosphorylation of brain MAP-2 conducted in vitro (36).

The maintenance of a minimal level of cAMP is required to support glucose-induced insulin secretion from fluorescence-activated cell sorter-purified \(\beta\)-cells (43, 44), and other studies have localized an effect of CAMP to potentiate Ca\(^{2+}\)-induced insulin secretion to some distal step of the secretory process (45). Since MAP-2 may also serve as a substrate for PKA (46) (independent of the activation of protein kinase A).

Accordingly, H-89 (5 \(\mu\)M) had only modest effects on CaM kinase II activity (15\% inhibition) (Fig. 4). 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 \(\mu\)M) was only modestly (22\%) reduced in the presence of H-89 (5 \(\mu\)M), a specific inhibitor of PKA (46) (Fig. 4). In the presence of basal concentrations of Ca\(^{2+}\) (0.05 \(\mu\)M), forskolin induced a significant phosphorylation of MAP-2 (160 \(\pm\) 13\% relative to control, \(p = 0.004\)), which was totally abrogated by the inclusion of 5 \(\mu\)M H-89 (Fig. 4). 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 \(\mu\)M) was only modestly (22\%) reduced in the presence of H-89, an effect that was not statistically significant (\(p = 0.48\)) (Fig. 4). Accordingly, H-89 (5 \(\mu\)M) had only modest effects on CaM kinase II activity in \(\beta\)TC3 cell homogenates or on CaM kinase II-mediated phosphorylation of purified MAP-2 in vitro (–15\% inhibition in either case, data not shown). These observations demonstrate that the activation of PKA is capable of inducing MAP-2 phosphorylation in permeabilized \(\beta\)TC3 cells. This activation may contribute, although not significantly, to MAP-2 phosphorylation induced by 5 \(\mu\)M Ca\(^{2+}\). A logical explanation is provided by the demonstrated presence in the \(\beta\)-cell of calmodulin-dependent adenylate cyclase and phosphodiesterase activities that could mediate Ca\(^{2+}\)-dependent modulations of intracellular cAMP concentrations (47).

Identification of Site-specific Phosphorylation of MAP-2 by Two-dimensional Phosphopeptide Mapping—Attempts to sup-
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 nonspecific effects of these compounds. Although 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, six major and several minor phosphorylation sites for CaM kinase II on purified brain MAP-2 were identified (Fig. 5A), which is consistent with previous reports (9). 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 2β isofrom recently demonstrated to be prominently expressed in β-cells (48). 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$^{2+}$ (Fig. 5B, arrowheads) as verified by cation exchange with in vitro generated phosphopeptides (Fig. 5C). 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 assterted by CaM kinase II-specific phosphorylation may also be conserved.

Comparison of phosphopeptide digests generated from MAP-2 phosphorylated in the presence of basal (0.05 μM) or stimulatory (5 μM) Ca$^{2+}$ concentrations revealed significant differences. A representative experiment is illustrated in Fig. 6. 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 (Fig. 6, large open circle). Interestingly, this spot corresponded to the site most responsive to in vitro phosphorylation by purified CaM kinase II (Fig. 5A) 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. 5A), but significant differences in phosphate incorporation into these 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, which suggests 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 phosphorylation of MAP-2 revealed 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. 6, cross symbol) implicating the action of a Ca$^{2+}$-dependent phosphatase, e.g. calcineurin, as has been previously reported (49, 50). Ca$^{2+}$ also induced the phosphorylation of sites of similar migration to major sites targeted by PKA in vitro (Fig. 6, indicated by “p”) that were clearly distinct from sites targeted by CaM kinase II (Fig. 7). This suggests that these may represent AMP-induced phosphorylation events consistent with the ability of II-89 to modestly inhibit MAP-2 phosphorylation. Alternatively, they could represent sites phosphorylated by other Ca$^{2+}$-sensitive protein kinases such as protein kinase C (51) or MAP kinase (52). 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 phosphorylation in β-cells

**Fig. 5.** 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 under “Experimental Procedures.” 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 by ascending chromatography in the vertical dimension. Cerenkov counts/min loaded onto thin-layer plates were 1000 cpm (A), 300 cpm each (B), and 300 cpm each (C). ● indicates 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). ○ indicates a phosphopeptide that, although present upon in situ stimulation, is not phosphorylated by CaM kinase IIα in vitro. TLC, thin-layer chromatography.

**Fig. 6.** 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 and to two-dimensional phosphopeptide mapping as described under “Experimental Procedures.” Cerenkov counts/min loaded onto each thin-layer plate was 500 cpm. The large and small ○ indicate major phosphopeptides identified as CaM kinase II-specific. Other phosphopeptides that increased are indicated by p, whereas + marks a phosphopeptide that decreased in response to treatment with elevated Ca$^{2+}$ (5 μM). TLC, thin-layer chromatography.
phosphate incorporation rather than the overall amount is the critical factor for the specific regulation of MAP-2 (14). 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 (20–22, 53). 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 were suggested to be MAPs, although not identified (54). The phosphorylation of MAP-2 by CaM kinase II and PKA leads, at least in vitro, to the increased disassembly of microtubules (19) possibly through microtubule domain “stiffening” as shown for the low molecular weight MAP, tau (55). 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 exocytic site as a result of changes in microtubule dynamics. Indeed such a role for Ca2+-dependent kinases in granule translocation has recently been obtained from video microscopy experiments in living β-cells (56) and is consistent with recent evidence that this enzyme acts at a site proximal to granule exocytosis (3). 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,2 suggest that this enzyme may be perfectly poised to regulate insulin secretion via the regulation of microtubule function and its association with secretory granules.

Acknowledgments—We thank Jill Meisenhelder and Tony Hunter of the Salk Institute, La Jolla, CA for technical assistance with the two-dimensional phosphopeptide maps.

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doi: 10.1074/jbc.272.43.27464

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