Ca\textsuperscript{2+}-dependent Dephosphorylation of Kinesin Heavy Chain on β-Granules in Pancreatic β-Cells

IMPLICATIONS FOR REGULATED β-GRANULE TRANSPORT AND INSULIN EXOCYTOSIS*

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The specific biochemical steps required for glucose-regulated insulin exocytosis from β-cells are not well defined. Elevation of glucose leads to increases in cytosolic [Ca\textsuperscript{2+}], and biphaseic release of insulin from both a readily releasable and a storage pool of β-granules. The effect of elevated [Ca\textsuperscript{2+}] on phosphorylation of isolated β-granule membrane proteins was evaluated, and the phosphorylation of four proteins was found to be altered by [Ca\textsuperscript{2+}]. One (a 18/20-kDa doublet) was a Ca\textsuperscript{2+}-dependent increase in phosphorylation, and, surprisingly, three others (138, 42, and 36 kDa) were Ca\textsuperscript{2+}-dependent dephosphorylations. The 138-kDa β-granule phosphoprotein was found to be kinesin heavy chain (KHC). At low levels of [Ca\textsuperscript{2+}], KHC was phosphorylated by casein kinase 2, but KHC was rapidly dephosphorylated by protein phosphatase 2B β (PP2Bβ) as [Ca\textsuperscript{2+}] increased. Inhibitors of PP2B specifically reduced the second, microtubule-dependent, phase of insulin secretion, suggesting that dephosphorylation of KHC was required for transport of β-granules from the storage pool to replenish the readily releasable pool of β-granules. This is distinct from synaptic vesicle exocytosis, because neurotransmitter release from synaptosomes did not require a Ca\textsuperscript{2+}-dependent KHC dephosphorylation. These results suggest a novel mechanism for regulating KHC function and β-granule transport in β-cells that is mediated by casein kinase 2 and PP2B. They also implicate a novel regulatory role for PP2B/calcineurin in the control of insulin secretion downstream of a rise in [Ca\textsuperscript{2+}].

Early events in glucose-induced insulin release involve generation of a series of signals derived from glucose metabolism that alter ion-channel fluxes and lead to a rise in cytosolic [Ca\textsuperscript{2+}], (1, 2). It has been presumed that increased cytosolic [Ca\textsuperscript{2+}] in β-cells is the major secondary signal that stimulates distal exocytotic secretory events (3). However, the means by which increased cytosolic [Ca\textsuperscript{2+}], induces β-granule transport from an intracellular storage pool to be docked at a pre-exocytotic site against the plasma membrane and then to promote β-granule membrane/plasma membrane fusion for the final exocytotic event are poorly understood. Moreover, regulatory factors besides cytosolic [Ca\textsuperscript{2+}], as well as certain facilitating proteins, are necessary to instigate glucose-regulated insulin exocytosis (2, 4).

Recently, there have been insights into the mechanism of insulin exocytosis, largely guided by advances in understanding mechanisms of synaptic vesicle exocytosis (3, 4). However, there is currently no convincing indication as to how secondary coupling signals such as [Ca\textsuperscript{2+}], influenced by changes in nutrient metabolism, communicate with the β-cell’s exocytotic apparatus to promote insulin release. Hence, questions remain as to how insulin exocytosis is actually controlled. This is a complex issue because there are several stages to insulin exocytosis, all of which contain potential regulatory sites to control insulin release (4). This study focuses on the mechanism by which transport of β-granules from an intracellular storage pool to a readily releasable pool of granules at the β-cell plasma membrane might be controlled.

Previous work suggested that β-granules are transported along microtubules, likely driven by ATP-dependent motors such as kinesin (5, 6). Many members of the kinesin superfamily are found in mammalian cells (7–9), including conventional kinesin, a heterotetramer consisting of two kinesin heavy chains (KHC)

* The abbreviations used are: KHC, kinesin heavy chain; KLC, kinesin light chain; CK1, casein kinase-1; CK2, casein kinase-2; GSK3, glycogen synthase kinase-3; PP1, phosphoprotein phosphatase-1; PP2A, phosphoprotein phosphatase-2A; PP2B, phosphoprotein phosphatase-2B; RKB, Krebs-Ringer buffer; GFP, green fluorescent protein; WT, wild type; PVD, polyvinylidene difluoride; CAIN, calcineurin-inhibitory peptide; AdV, adenovirus; BSA, bovine serum albumin; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid.

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quences of this phosphorylation are uncertain. Effects on vesicle binding, ATPase activity, and microtubule binding have been reported (10, 13, 19–21), but in each case contradictory experimental evidence also exists (17, 18, 21, 22). Nevertheless, the fact that kinesin is a phosphoprotein in vivo suggests that phosphorylation of specific residues on kinesin by specific kinases is likely to be important for regulating some aspects of kinesin function, just as the predominant regulation of myosins and dyneins is through phosphorylation (23, 24). The key will be to identify specific kinases and phosphatases important for regulation of a given kinesin-dependent process in vivo.

Several protein kinases have been implicated in stimulus-coupling mechanisms for glucose-induced insulin release (25). In particular, activation of Ca\(^{2+}\)/calmodulin-dependent kinase-2 correlates with glucose-induced insulin exocytosis, most likely in response to nutrient-induced rises in β-cell cytosolic [Ca\(^{2+}\)], (26). In addition, certain protein kinase C isoforms appear to be involved in nutrient-regulated insulin exocytosis, presumably as a result of nutrient-induced rises in both cytosolic long chain acyl-CoA and [Ca\(^{2+}\)], (2, 27). Likewise, protein kinase A is important for the potentiation of nutrient-induced insulin release by incretins such as glucagon-like peptide 1 and glucose-dependent insulinotropic peptide (28). To counterbalance protein kinase activities, phosphoprotein phosphatases must also play a role in the nutrient-mediated regulation of insulin release in β-cells, even though this has not been extensively investigated. Regardless, there is little information about the appropriate protein kinase/phosphatase substrates relevant to the insulin exocytosis mechanism, nor is there much known about how the phosphorylation state of such protein substrates could control insulin release.

In this study, we present evidence that kinesin heavy chains in β-granules are phosphorylated under basal condition in β-cells by casein kinase-2 (CK2), and dephosphorylated by phosphoprotein-2B (PP2B, also known as calcineurin) in a Ca\(^{2+}\)-dependent manner under conditions that stimulate insulin secretion. This represents a suitable “exocytotic phosphoprotein substrate” downstream of a rise in [Ca\(^{2+}\)], not previously documented in β-cells. This study highlights a novel regulatory aspect of the insulin exocytotic mechanism, whereby activation of Ca\(^{2+}\)-dependent dephosphorylation of kinesin controls sustained β-granule transport.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture medium, unless otherwise noted, was supplied by Invitrogen. Fetal bovine serum was obtained from HyClone Laboratories, Inc. (Logan, UT). The \(^{32}\)Porthophosphate (10 mCi/ml orthophosphate in aqueous solution), adenosine 5’-(γ-\(^{32}\)P)triphosphate (tritylammomium salt, 5000 Ci/mmol), 3’-(\(^{32}\)iodo)tyrosyl insulin (human recombinant, 2000 Ci/mmoll), Protein A-Sepharose, and Percol\(^{TM}\) were purchased from Amersham Biosciences. Accuendenz\(^{TM}\) was obtained from the Accurate Chemical and Scientific Corp. (Westbury, NY). The MAPS II purification kit was obtained from Bio-Rad. GSK3-α/β antibody and protein phosphatase antibodies against the catalytic subunit of PP1, PP2A, and PP2B (α- and β-isoforms) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-Erk-1/-2 antisemur was from Promega Corp. (Madison, WI), and total Erk-1/-2 antisemur was a gift from Dr. M. Cobb (University of Texas Southwestern Medical Center, Dallas, TX). Anti-rabbit and anti-sheep IgG horseradish peroxidase conjugates were from Jackson Immunoresearch (West Grove, PA). The monoclonal antibody against the kinesin motor domain, SUK-4, was obtained from Covance Inc. (Princeton, NJ). The FLAG antisemur was purchased from Sigma. Various kinase, phosphatase inhibitors, and peptide substrates were obtained from Calbiochem (San Diego, CA), unless otherwise stated. The anti-mouse IgG horseradish peroxidase conjugate was from Upstate Biotechnology, Inc. (Lake Placid, NY).

**In vitro** \(\text{Ca}^{2+}\)-dependent dephosphorylation of β-granule proteins. A highly enriched β-granule fraction was prepared from rat insulinoma tissue and subjected to an \textit{in vitro} phosphorylation assay as described under “Experimental Procedures.” Panel A shows a typical autoradiograph analysis done in the absence of [Ca\(^{2+}\)] (10 mM EGTA added to chelate endogenous [Ca\(^{2+}\)], or with 20 μM or 2 mM buffered free [Ca\(^{2+}\)]. Six β-granule phosphoproteins are indicated and labeled according to their apparent molecular weight: p138, p50, p42, p36, and p18/p20 doublet. Panel B shows example autoradiograph analysis of the Ca\(^{2+}\) dependence of the phosphorylation of these β-granule proteins. Essentially the β-granule in \textit{in vitro} phosphorylation was conducted standard Ca\(^{2+}\)-buffered solutions to give free [Ca\(^{2+}\)] from pCa 2 to pCa 8 (World Precision Instruments Inc.) (34), as described under “Experimental Procedures.” From a series of such titration experiments (n = 3), an estimated \(K_{d}\), of [Ca\(^{2+}\)] for (de)phosphorylation of β-granule proteins could be obtained.

by collagenase digestion, followed by Histopaque-Ficol\(^{TM}\) density gradient centrifugation as previously described (29).

The pancreatic β-cell line, INS-1 (between passages 60 and 70), was maintained in the complete medium RPMI 1640 (11.2 mM glucose) containing 10% (v/v) fetal calf serum, 50 μM β-mercaptoethanol, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and incubated at 37 °C, 5% CO\(_2\) as previously described (30).

**Insulinoma Propagation and Subcellular Fractionation**—Transplantable rat insulinoma tissue was propagated in NDEH rats as previously described (31, 32). Insulinoma subcellular fractions highly enriched in insulin secretory granules, plasma membrane and cytosol, were prepared by differential, Accuendenz\(^{TM}\) discontinuous density gradient and Percoll\(^{TM}\) continuous density centrifugations and monitored by marker enzyme analysis as previously described (32, 33).

**β-Granule in Vitro Protein Phosphorylation Assay**—The highly en-
Ca²⁺-dependent Kinesin Dephosphorylation on β-Granules

FIG. 2. Identification of the p138 β-granule phosphoprotein as KHC. In panel A, the β-granule in vitro phosphorylation assay was carried out with two separate β-granule fractions (ISG-1 and ISG-2) in the absence (10 mM EGTA) or presence of 2 mM [Ca²⁺] as described under “Experimental Procedures.” Phosphoproteins were resolved by gel electrophoresis and transferred onto a PVDF membrane for immunoblot analysis.

Phospho-KHC was then subjected to autoradiography to detect phosphorylation reaction initiated by addition of 1 μCi of [γ-²²P]ATP for 15 min at 34 °C. The total volume of the reaction was 20 μl. After incubation, the reaction was stopped with one volume of Laemmli buffer and the reactions run on a 7.5–17% polyacrylamide gel. The gel was then dried and subjected to autoradiography and PhosphorImager analysis.

Phosphate Loading of Pancreatic Islet β-Cells—For [²²P]phosphate loading of pancreatic β-cells, either 200 isolated rat islets or INS-1 cells (70% confluence on 10-cm² tissue culture dishes) were incubated in RPMI 1640 medium as described above, but containing 2.8 mM glucose, at 37 °C for 16–20 h. The cells/islets were then washed twice in phosphate-free RPMI 1640 medium and then incubated for 4 h in 800 μl of the same medium also containing 100 μCi/ml [²²P]phosphate. INS-1 cells/islets were then washed twice in Krebs-Ringer buffer (KRB) containing 2.8 mM glucose and 0.1% BSA (w/v). INS-1 cells were pre-incubated in 1 ml, and islets in 200 μl, of 2.8 mM glucose KRB for 30 min at 37 °C, then incubated for another 30 min in the same volume of KRB plus 0.1% BSA containing 2.8 mM glucose; 16.7 mM glucose; or 16.7 mM glucose, 10 μM forskolin, 1 mM isobutylmethylxanthine, 30 mM KCl, and 10 μM phorbol 12-myristate 13-acetate to render β-cells with a full stimulation of insulin exocytosis (35). This latter addition is colloquially named the “Boston mixture.” After this second incubation at 37 °C, the medium was removed and the cells/islets were then washed twice in ice-cold phosphate-buffered saline. One ml of ice-cold lysis buffer (20 mM Hepes (pH 7.4), 200 mM NaCl, 10 mM β-glycerol phosphate, 10 mM NaF, 20 mM sodium pyrophosphate, 10 mM EDTA, 400 μM sodium orthovanadate, 20 mM sodium orthovanadate, 1 mM isobutylmethylxanthine, 30 mM KCl, and 10 μM phorbol 12-myristate 13-acetate) was added to the KRB or the KRB plus 100 mM CaCl₂ (to give a buffered [Ca²⁺] of 2 μM), or in standard Ca²⁺-buffered solutions to give free [Ca²⁺] ranging from 2 to 20 μM.

Synaptosome [²²P]Orthophosphate Labeling—Four freshly dissected rat brain cortices were used as starting material. Synaptosomes were prepared as described previously (36). Synaptosomes were equilibrated in modified Krebs buffer (20 mM Hepes (pH 7.4), 1.2 mM MgCl₂, 0.1 mM CaCl₂, 11 mM glucose, 128 mM NaCl, 3 mM KCl), centrifuged at 12,000 × g for 20 min, and resuspended in 5 ml of the Krebs buffer. [²²P]Orthophosphate (0.5 mM) was then added and incubated for 40 min at 37 °C. Five 1-ml aliquots of the synaptosome suspension were centrifuged at 10,000 × g in a microcentrifuge tube for 1 min. The supernatant was discarded and synaptosomes resuspended in 0.5 ml of Krebs buffer (for no depolarization condition, also considered “time 0”) or in 0.5 ml “depolarization buffer” (20 mM Hepes (pH 7.4), 1.2 mM MgCl₂, 0.1 mM CaCl₂, 11 mM glucose, 16.5 mM NaCl, 117 mM KCl), and incubated for 15, 30, and 90 s at 37 °C. Synaptosomes were lysed by adding one volume of 2× lysis buffer supplemented with 50 mM okadaic acid, 0.2 mM sodium orthovanadate, and 50 mM microcystin LR. After 90 min of lysis, the samples were then centrifuged at 10,000 × g for 2 min to remove membranes and unincorporated orthophosphate, and then the supernatants were analyzed by gel electrophoresis and the gels were dried for autoradiography.

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In Vitro Phosphorylation of Kinesin—Rat brain kinesin was purified as described previously (22). Recombinant CK2 (50 units; New England Biolabs) were incubated with and without 40 ng of purified kinesin in IME buffer (15 mM imidazole, 2 mM MgCl₂, and 1 mM EDTA) supplemented with 0.1 mM ATP, 2 mM MgCl₂, and 500 μCi/μmol [γ-²²P]ATP for 15 min at 34 °C. The total volume of the reaction was 20 μl. After incubation, the reaction was stopped with one volume of Laemmli buffer and the reactions run on a 7.5–17% SDS-PAGE gel. The gel was then dried and subjected to autoradiography and PhosphorImager analysis.

Phosphorylation of pancreatic islet β-cells was conducted in modified Krebs buffer containing 16.7 mM glucose, 0.1 mM CaCl₂, 11 mM glucose, 128 mM NaCl, 3 mM KCl, and 10 μM phorbol 12-myristate 13-acetate to render β-cells with a full stimulation of insulin exocytosis (35). This latter addition is colloquially named the “Boston mixture.” After this second incubation at 37 °C, the medium was removed and the cells/islets were then washed twice in ice-cold phosphate-buffered saline. One ml of ice-cold lysis buffer (20 mM Hepes (pH 7.4), 200 mM NaCl, 10 mM β-glycerol phosphate, 10 mM NaF, 20 mM sodium pyrophosphate, 10 mM EDTA, 400 μM sodium orthovanadate, 20 mM sodium orthovanadate, 1 mM isobutylmethylxanthine, 30 mM KCl, and 10 μM phorbol 12-myristate 13-acetate) was added to the KRB or the KRB plus 100 mM CaCl₂ (to give a buffered [Ca²⁺] of 2 μM), or in standard Ca²⁺-buffered solutions to give free [Ca²⁺] ranging from 2 to 20 μM. After this second incubation at 37 °C, the medium was removed and the cells/islets were then washed twice in ice-cold phosphate-buffered saline. One ml of ice-cold lysis buffer (20 mM Hepes (pH 7.4), 200 mM NaCl, 10 mM β-glycerol phosphate, 10 mM NaF, 20 mM sodium pyrophosphate, 10 mM EDTA, 400 μM sodium orthovanadate, 20 mM sodium orthovanadate, 1 mM isobutylmethylxanthine, 30 mM KCl, and 10 μM phorbol 12-myristate 13-acetate) was added to the cells/islets. Cells/islets were transferred to 1.5-ml microcentrifuge tubes and sonicated on ice islets (25 watts; 10 s). The lysates were then subjected to immunoprecipitation.

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rotary incubation at 4 °C, lysates were centrifuged at 10,000 × g for 15 min in a microcentrifuge. Supernatants were removed, and kinesin, synapsin, and dynamin were sequentially immunoprecipitated or affinity-purified from lysates as described below using anti-kinesin monoclonal antibodies, 5 μg of anti-synapsin I antibody (Serotec), or glutathione S-transferase-Grb2 bound to glutathione-Sepharose beads. Samples were run on a 7.5–16% SDS-PAGE gel. The gel was then dried and subjected to autoradiography and PhosphorImager analysis.

Kinesin Heavy Chain Immunoprecipitation—A mix of monoclonal antibodies against kinesin heavy (H2) and light (L2, 63–90, and KLC-all) chains were covalently cross-linked to Protein A-agarose beads using dimethylpimelimidate at a cross-linking ratio of ~2 mg of IgG/ml of bed volume. For control beads, normal mouse IgG was linked using the same procedure. Kinesin immunoprecipitations on 32P-labeled islet/INS-1 cell or synaptosomes lysates were applied to examine the phosphorylation state of kinesin. Lysates were centrifuged at 10,000 × g for 5 min at 4 °C to remove cellular debris. The supernatant was removed and pre-cleared by incubating with 20 μl of a 50% (w/v) slurry of Protein A-conjugated Sepharose beads in phosphate-buffered saline for 60 min.

Samples were centrifuged at 3000 × g for 30 s, and the supernatant was transferred to microcentrifuge tubes containing 20 μl of a 50% (w/v) slurry of kinesin antibodies conjugated to agarose and then incubated with rotary mixing overnight at 4 °C. The anti-kinesin agarose beads were then pelleted by centrifugation (30 s; 3000; 4 °C). The supernatant was removed, and the beads washed twice in wash buffer I (20 mM Hepes, 0.1% (w/v) CHAPS, 0.5% (w/v) Me2SO) as control in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum and 5.6 mM glucose. The supernatant was then transferred to SwinnexTM chambers (Millipore, Bedford, MA; 125 μm polycarbonate hydrophilic filters) and pre-cleared by incubating with 20 μl of a 50% (w/v) slurry of Protein A-agarose beads in phosphate-buffered saline for 60 min. The beads were then washed twice in lysis buffer to remove excess salt. The supernatant was aspirated, and the beads washed were twice in wash buffer II (20 mM Hepes, 0.5% (v/v) Triton X-100, 1% (w/v) EDTA, 100 mM NaF, 4 mM EDTA, 10 μg/ml aprotinin, 100 μM phenylmethylsulfonyl fluoride) followed by sonications (25 watts; 10 s). The fully labeled supernatant was aspirated, and the beads were incubated with recombinant CK2 in the presence of [γ-32P]ATP. The samples were separated by SDS-PAGE, and gels were dried and analyzed by PhosphorImager. Positions of CK2 phosphorylated subunits are indicated. CK2 phosphorylated both KHC and KLC in vitro.
were cultured overnight (43). For adenovirus-mediated protein expression, isolated rat islets green fluorescent protein (GFP) was generated as previously described (41, 42). A control recombinant adenovirus expressing calcineurin-inhibitory peptide (CAIN) (40), were generated as previously described (39). For p138 phosphorylation assay, the adenovirus in vitro phosphorylation assay was carried out in the absence (10 mM EGTA) or presence of 2 mM [Ca^{2+}] (10 mM EGTA + 12 mM CaCl_2) as described under “Experimental Procedures” in the presence of nonimmune rabbit antisera (control), or blocking antibodies against PP1, PP2A, or PP2B (α- and β-isoform catalytic subunits). An example autoradiograph analysis for p138/KHC (de)phosphorylation of at least three individual experiments is shown.

Other Procedures—Protein assay was by the bieinchoninic acid method (Pierce). Data are presented as means ± S.E. Statistically significant differences between groups were analyzed using Student’s t test, where p < 0.05 was considered statistically significant.

RESULTS

Ca^{2+}-dependent Phosphorylation and Dephosphorylation of β-Granule Proteins—A highly enriched β-granule fraction was examined for endogenous Ca^{2+}-dependent protein phosphorylation in vitro. A number of phosphoproteins were detected that had varied responses to [Ca^{2+}] in terms of their phosphorylation state (Fig. 1). A 50-kDa ß-granule protein (p50) was phosphorylated, but in a Ca^{2+}-independent manner (Fig. 1A). However, an 18/20-kDa doublet (p18/p20) had a significantly increased phosphorylation state in the presence of Ca^{2+} (p ≤ 0.05): 2.8 ± 0.4-fold (n = 4) at 20 μM [Ca^{2+}], and 3.6 ± 0.5-fold (n = 4) at 2 mM [Ca^{2+}] above that in the absence of exogenous Ca^{2+} (i.e. 10 mM EGTA; Fig. 1A). Ca^{2+} titration curves were constructed in standard Ca^{2+}-buffered solutions to give free [Ca^{2+}] from pCa 2 to pCa 8 (World Precision Instruments Inc.) (34). The p18/p20 phosphorylation increased with increasing [Ca^{2+}] that was sustained above 1 mM [Ca^{2+}] that was sustained above 1 mM [Ca^{2+}] [Ca^{2+}] (10 mM EGTA; Fig. 1B). In contrast, three other β-granule phosphoproteins of 138 (p138), 42 (p42), and 36 kDa (p36) were significantly dephosphorylated in a Ca^{2+}-dependent manner (p ≤ 0.05). For p138, 15.4 ± 2.1% (n = 4) at 20 μM [Ca^{2+}], and 12.7 ± 1.9% (n = 4) at 2 mM [Ca^{2+}] of the p138 phosphorylation state in absence of exogenous Ca^{2+} was observed; for p42, 13.2 ± 1.5% (n = 4) at 20 μM [Ca^{2+}], and 8.9 ± 1.0% (n = 4) at 2 mM [Ca^{2+}] of the p42 phosphorylation state in absence of exogenous Ca^{2+} was found; and for p36, it was 20.2 ± 2.4% (n = 4) at 20 μM [Ca^{2+}], and 12.4 ± 0.9% (n = 4) at 2 mM [Ca^{2+}] of the p36 phosphorylation state in absence of Ca^{2+} (Fig. 1A). Ca^{2+} titration curves indicated that p138 phosphorylation decreased with increasing [Ca^{2+}] (K_{i(0.5)} between 0.1 and 1.0 μM [Ca^{2+}]), reaching a maximum dephosphorylation >5 μM [Ca^{2+}] (Fig. 1B). Likewise, p42 phosphorylation decreased with increasing [Ca^{2+}] (K_{i(0.5)} between 0.1 and 1.0 μM [Ca^{2+}]), reaching a maximum dephosphorylation state >100 μM [Ca^{2+}] (Fig. 1B). For p36, phosphorylation also decreased with increasing [Ca^{2+}] (K_{i(0.5)} between 0.1 and 1.0 μM [Ca^{2+}]), reaching a maximum dephosphorylation state >100 μM [Ca^{2+}] (Fig. 1B).

To determine whether β-granule phosphoproteins were soluble or membrane associated proteins, fractions were centrifuged. After the in vitro [32P]phosphorylation assay, the β-granule fraction was osmotically lysed by dilution in 500 μl of 10 mM ammonium persulfate (pH 9.0) and incubation on ice for 30 min. These samples were subjected to 100,000 × g at 4 °C for 60 min (SW55 rotor, Beckman LE-80 ultracentrifuge) to pellet a β-granule membrane fraction. Pellets were resuspended in electrophoresis sample buffer; supernatants were lyophilized and then resuspended in electrophoresis sample buffer. Subse-

![Graph](http://example.com/graph.png)
quent electrophoresis and autoradiography revealed that p138, p50, p42, p36, and p18/p20 were all in pellet fractions, indicating that these were β-granule membrane-associated proteins. Immunoblot analysis of β-granule fractions for proinsulin endopeptidase PC2 (29) revealed >90% of this β-granule matrix protein to be in β-granule soluble fractions, confirming separation of β-granule membrane-associated and soluble components (data not shown).

Identification of p138 β-Granule Phosphoprotein as a KHC—

The identity of most β-granule phosphoproteins in Fig. 1 is yet to be determined, with the exception of p138, which had a molecular weight similar to that of kinesin heavy chain. After β-granule in vitro 32P-phosphorylation, β-granule proteins were resolved on SDS-PAGE and transferred to a PVDF membrane. The Ca2+-dependent dephosphorylation of p138 was observed by autoradiography of the PVDF membrane (Fig. 2A, left panel) and subjected to immunoblot analysis for KHC (Fig. 2A, right panel). These two analyses did not interfere with each other. The p138 phosphorylated and 138-kDa KHC immunoreactive bands on the same gel were superimposable (Fig. 2A). The total amount of KHC immunoreactivity did not vary between samples, reaffirming the specific Ca2+-dependent nature of p138/KHC dephosphorylation rather than a reflection of changes in protein loading (Fig. 2A). Preincubation of β-granules with a function blocking KHC specific antibody, SUK4 (45), inhibited phosphorylation of p138/KHC (Fig. 2B), thereby confirming identification of p138 as KHC. SUK4 preincubation did not affect phosphorylation or Ca2+-dependent dephosphorylation of p42 and p36 on the same autoradiograph analysis (Fig. 2B).

CK2 Phosphorylates β-Granule KHC—Assaying β-granule in vitro phosphorylation in the presence of various protein kinase inhibitors gave an indication of the protein kinase responsible for phosphorylating KHC in pancreatic β-cells. Inhibitors of protein kinase A (e.g. H-89), protein kinase C (e.g. bisindolylmaleimide), or Ca2+/calmodulin-dependent kinase-2 (e.g. KN-93), all of which were previously implicated in the control of insulin secretion (46), had no effect on β-granule p138/KHC phosphorylation (data not shown). Likewise, inhibition of tyrosine kinases (e.g. genistein) had no effect on p138/KHC phosphorylation. A casein kinase-1 (CK1) peptide substrate used as a competitive inhibitor in the β-granule phosphorylation assay had no effect on p138/KHC, p42, or p36 phosphorylation (Fig. 3A), whereas a CK2 peptide substrate used as a competitive inhibitor markedly inhibited p138/KHC phosphorylation (>90% at concentrations ≥5 μM compared with the control in the absence of [Ca2+]). The CK2 peptide also inhibited p42 phosphorylation to a lesser extent (Fig. 3B), but appeared to have no effect on p36 phosphorylation (Fig. 3B). A GSK3 blocking antibody had no effect on p138/KHC or p42 phosphorylation in β-granules as compared with control containing an equivalent 5-μl amount of nonimmune serum (Fig. 3C), but the GSK3 antibody prevented p36 phosphorylation in the absence of [Ca2+]i (Fig. 3C). In summary, an extensive analysis with numerous protein kinase inhibitors indicated that β-granule p138/KHC (and probably p42) were phosphorylation substrates for CK2, and that p36 was a phosphorylation substrate for GSK3α/β (Fig. 3).

The ability of CK2 inhibitors to inhibit phosphorylation of kinesin in β-granule fractions suggested a role for CK2, but could not determine whether CK2 phosphorylated kinesin heavy chains directly. To demonstrate direct phosphorylation of kinesin by CK2, purified, native rat brain kinesin was used for in vitro phosphorylation experiments with recombinant CK2 (Fig. 4). When purified kinesin was incubated with recombinant CK2 in the presence of radiolabeled ATP, both heavy and light chains of kinesin were heavily phosphorylated. No incorporation was observed when purified kinesin alone was incubated with ATP (data not shown). These results strongly suggest that phosphorylation of kinesin heavy chain in β-granule was the result of a direct action of CK2 on kinesin. PP2B Dephosphorylates β-Granule KHC in a Ca2+/Calmodulin-dependent Manner—Increased [Ca2+]i decreased phosphorylation of β-granule p138/KHC, p42, and p36 (Figs. 1–3), suggesting a role for the Ca2+/calmodulin-dependent PP2B (also known as calcineurin). This possibility was evaluated for p138/KHC dephosphorylation by using phosphoprotein phosphatase inhibitors in the in vitro β-granule phosphorylation assay (Fig. 5). The generic phosphatase inhibitor, NaF, had no effect on p138/KHC dephosphorylation (Fig. 5). The general phosphatase competitive inhibitor pyrophosphate tended to inhibit p138/KHC dephosphorylation at concentration ≥1 mM, whereas protein-tyrosine phosphatase inhibitors (orthovandate and dephostatin) had no effect (Fig. 5). Okadaic acid and endothall preferentially inhibited phosphoprotein phosphatase-1 (PP1), but also inhibited phosphoprotein phosphatase-2A (PP2A) at >100 nM concentrations. However, neither of these reagents affected β-granule p138/KHC dephosphorylation (Fig. 5). More specific inhibitors of PP2A (cantharidin and calyculin-A) also had no effect on β-granule Ca2+-dependent p138/KHC dephosphorylation (Fig. 5). In contrast, the
PP2B-specific inhibitor, cypermethrin, inhibited β-granule Ca\(^{2+}\)-dependent p138/KHC dephosphorylation at concentrations ≥500 nM, as well as increasing p138/KHC phosphorylation levels, a characteristic of appropriate phosphatase inhibition (Fig. 5). The calmodulin antagonist, calmidazolium, also inhibited β-granule p138/KHC dephosphorylation and enhanced its phosphorylation state at concentrations ≥10 μM, consistent with the dependence of PP2B Ca\(^{2+}\)-dependent phosphatase on calmodulin (Fig. 5). The effects of specific phosphoprotein phosphatase antisera on Ca\(^{2+}\)-dependent p138/KHC dephosphorylation were also examined in the β-granule in vitro phosphorylation assay. PP1 and PP2A antisera had no effect, whereas PP2B completely inhibited β-granule Ca\(^{2+}\)-dependent p138/KHC dephosphorylation (Fig. 6). In summary, phosphoprotein phosphatase inhibitor and blocking antibody studies indicated the Ca\(^{2+}\)/calmodulin PP2B phosphatase isozyme was enriched in the cytosolic fraction (Fig. 7A) and indeed PP1 was appreciably decreased in the β-granule fraction (Fig. 7B). The PP2B α-isozyme was not detectably expressed in pancreatic β-cells, despite significant expression in rat brain (Fig. 7B). However, the PP2B β-isozyme was expressed in insulinoma β-cells, enriched on β-granules, and reduced in the cytosolic fraction (Fig. 7B). In summary, CK2 and PP2Bβ are located on β-granules consistent with CK2-mediated phosphorylation of KHC and subsequent Ca\(^{2+}\)/calmodulin-dependent PP2B phosphatase activity (Fig. 3C). In addition, enrichment of GSK3β on β-granules is consistent with GSK3-mediated phosphorylation of β-granule p36 (Fig. 3C).

**Dephosphorylation of Kinesin Heavy Chain in Isolated Pancreatic Rat Islets Was Inversely Proportional to Glucose-induced Insulin Release**—To ascertain whether KHC phosphorylation was regulated under physiological stimulation of primary β-cells, isolated rat pancreatic islets were loaded with \(^{32}\)Porthophosphate and then incubated for 1 h at a basal 2.8 mM glucose, a stimulatory 16.7 mM glucose, or with a mixture intended to give a maximum stimulation of insulin exocytosis (35). Subsequent immunoprecipitation of KHC indicated that its \(^{32}\)Pphosphorylation state decreased with increasing glucose concentration and maximal stimulation of insulin secretion (Fig. 8A). This KHC dephosphorylation was specific and not necessarily because of a nonspecific depletion of intracellular ATP pools. In the same islets glucose-induced dephosphorylation of the MAP-kinases Erk-1/2 was observed as determined by phospho-Erk-1/2 immunoblot analysis (Fig. 8B), without

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**Fig. 8. In vivo phosphorylation of pancreatic islet KHC inversely correlates with stimulated insulin secretion.** Isolated pancreatic islets were loaded with \(^{32}\)Porthophosphate as described under “Experimental Procedures,” then incubated for 30 min at a basal 2.8 mM glucose, stimulatory 16.7 mM glucose, or a collection of secretagogues containing 16.7 mM glucose, 10 μM forskolin, 1 mM isobutylmethylxanthine, 30 mM KCl, and 10 μM phorbol 12-myristate 13-acetate (named the Boston mixture (Boston Cocktail) designed to give a maximum stimulation of insulin exocytosis (28). Panel A, \(^{32}\)P/KHC was immunoprecipitated and analyzed by autoradiography as described (see “Experimental Procedures”), with immunoblot (IB) analysis of the immunoprecipitates indicating that equivalent amount of KHC was immunoprecipitated. An example autoradiograph is shown. Panel B, immunoblot analysis of Erk-1/2 phosphorylation (as described under “Experimental Procedures”) in the same islets as in panel A with immunoblot analysis of total Erk-1/2 indicating equivalent levels present in the islets analyzed. Panel C, in parallel experiments the degree of insulin secretion from isolated pancreatic islets incubated under the same conditions as in panels A and B was determined as described (see “Experimental Procedures”). A mean ± S.E. of at least three individual experiments carried out in duplicate is shown.
Ca\textsuperscript{2+}-dependent Kinesin Dephosphorylation on \(\beta\)-Granules

Phosphorylation of Kinesin Is Not Linked to Release of Neurotransmitter from Synaptosomes—The data derived from \(\beta\)-cells suggested that the phosphorylation/dephosphorylation of kinesin heavy chain by CK2 and PP2B plays an important role in the second phase of glucose-stimulated insulin exocytosis. To determine whether this was a general feature of other exocytotic processes, we examined the phosphorylation status of kinesin during depolarization-induced release of neurotransmitter from purified, functional synaptosomes. This preparation has been extensively characterized at the ultrastructural, biochemical, and functional level (22). Both phosphorylation and dephosphorylation of neuronal proteins have been well characterized during depolarization-induced exocytosis. Synapsin I, for example, undergoes a rapid increase in phosphorylation caused by the actions of calmodulin kinase and/or protein kinase A, followed by a slow dephosphorylation (48). In contrast, dynamin concurrently undergoes a pronounced dephosphorylation (49). Both events were observed during depolarization-induced exocytosis in our preparations (Fig. 11). In contrast, kinesin phosphorylation was unaffected by depolarization-induced secretion from synaptosomes (Fig. 11). No detectable changes in phosphorylation state of either KHC or KLC were seen, although both kinases and phosphatases were being activated in the synaptosome indicated by synapsin phosphorylation and dynamin dephosphorylation (Fig. 11).

DISCUSSION

Kinesin is known to be present in \(\beta\)-cells (5), and suppression of kinesin expression by antisense oligonucleotide treatment reduced glucose-induced insulin secretion (6). Kinesin plays a role in attaching granules to microtubulin and as an ATP-dependent motor driving \(\beta\)-granules along microtubules toward the plasma membrane (6). However, it should be considered that \(\beta\)-granule transport is tightly controlled in the \(\beta\)-cell to appropriately replenish the ready releasable pool of \(\beta\)-granules.
at the plasma membrane lost by glucose-induced exocytosis (4). As such, it is probable that kinesin is regulated in the β-cell under the influence of glucose. However, it is unclear how kinesin activity is controlled in β-cells. Kinesin regulation, in part, appears to be characteristic of the cell type and/or kinesin isoform present, and possibly by its phosphorylation state and/or associated proteins (10–22). In this study, immunoblot analysis could not detect KLC associated with the KHC on β-granules (data not shown), and as such it was thought that kinesin activity was more likely regulated by KHC phosphorylation.

Although phosphorylation of β-granule proteins was demonstrated previously and proposed to play a role in insulin secretion (50, 51), the identity of relevant phosphoprotein substrates is largely unknown. Furthermore, the intrinsic Ca2+-dependent phosphorylation of β-granule proteins has not been examined. An in vitro phosphorylation assay revealed that the phosphorylation state of at least four β-granule proteins was regulated in a Ca2+-dependent manner (Fig. 1). Surprisingly, increased [Ca2+]i caused dephosphorylation of three of these proteins, suggesting an important role for the Ca2+/calmodulin-dependent PP2B (also known as calcineurin) in control of insulin exocytosis. The identity of the p18/p20, p36, and p42 β-granule phosphoproteins remains unknown, but immunological and pharmacological data identified p138 as KHC. Further analysis indicated that β-granule KHC was phosphorylated by CK2 and confirmed it to be dephosphorylated in a Ca2+-/calmodulin-dependent manner by PP2Bβ. Moreover, all three components (CK2, PP2B β-isofrom, and KHC) were enriched in β-granule fractions. Calmodulin was previously shown to be associated with β-granules in pancreatic β-cells (52), as required for Ca2+-dependent PP2B activation (53). Thus, relevant kinase and phosphatase activities were present in the same β-cell intracellular compartment as their KHC substrate. Significantly, this effect of phosphorylation and dephosphorylation of KHC was not seen in synaptosomes, suggesting that this may be a mechanism specific to secretory cells with large microtubule-associated storage pools.

Glucose is the most physiologically relevant nutrient controlling insulin secretion from pancreatic β-cells (1, 2). Elevated extracellular glucose levels result in increased glucose metabolism in β-cells, leading to a rise in the intracellular ATP/ADP ratio that then causes closure of KATP channels, depolarization, then opening of voltage-sensitive L-type Ca2+-channels and a subsequent increase in cytosolic [Ca2+]i (1, 2). The rise in [Ca2+]i is an important contributing factor to triggering both

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**Figure 10.** Adenoviral-mediated expression of the specific phosphoprotein phosphatase-2B inhibitor, CAIN, in isolated islets inhibits glucose-induced dephosphorylation of kinesin and insulin secretion. Isolated rat pancreatic islets were infected with recombinant adenoviruses (∼106 plaque-forming units/ml) that express GFP (AdV-GFP), native PP2Bα (AdV-Cn-WT), a constitutively active PP2Bα variant (AdV-CnA), or the PP2B-specific inhibitor, CAIN (AdV-CAIN), as described (see "Experimental Procedures"). Glucose-induced insulin secretion was evaluated over a 1-h incubation at 37 °C, and Erk-1/2 phosphorylation and KHC dephosphorylation in [32P]orthophosphate-loaded adenovirus-infected islets were examined as described (see "Experimental Procedures"). Panel A, PP-2Bα and FLAG epitope (for CAIN expression) immunoblot analysis of adenovirus-mediated protein expression in isolated islets. Panel B, glucose-induced insulin secretion in adenovirus-infected islets where the data are expressed as a mean ± S.E. of at least six individual experiments, and asterisk indicates statistically significant difference or p ≤ 0.02 compared with AdV-GFP-infected control islets. Panel C, immunoprecipitated (IP) [32P]KHC analyzed by autoradiography with immunoblot (IB) analysis of the immunoprecipitates indicating that equivalent amount of KHC was immunoprecipitated. An example autoradiograph is shown. Panel D, immunoblot analysis of Erk-1/2 phosphorylation in the same islets as in panel D with immunoblot analysis of total Erk-1/2 indicating equivalent levels present in the islets analyzed.
insulin release (3) and β-granule transport (4). It is therefore proposed that at basal glucose concentrations, cytosolic [Ca^{2+}] is relatively low, PP2B will be comparatively inactive, and KHC phosphorylation by constitutively active CK2 is high. As a consequence, kinesin activity and β-granule transport would be low. In contrast, cytosolic [Ca^{2+}] is markedly increased at stimulatory glucose concentrations (2, 3), so that PP2B would be activated and β-granule KHC dephosphorylated. As a result, kinesin ATP-dependent motor activity is activated and β-granule transport along microtubules triggered (4). Such a scenario is supported by our observations that the KHC phosphorylation state in pancreatic β-cells is inversely proportional to glucose-stimulated insulin secretion (Figs. 2 and 4) and that only the second phase of the biphasic insulin secretory response to a stimulatory glucose concentration was significantly affected.

The first phase of release represents the ready releasable pool of β-granules already docked at the β-cell plasma membrane and does not require additional movement along microtubules. In contrast, the second phase requires mobilization of β-granules from a storage pool to replenish the readily releasable pool of β-granules in addition to β-granule docking and the final stages of stimulated exocytosis (4). Disruption of microtubules in islet β-cells preferentially inhibits this second phase of insulin release, indicating that microtubules are required for β-granule transport during this stage (54, 55). In this study, specific inhibition of PP2B in pancreatic islet β-cells by AdV-CAIN or cypermethrin significantly inhibited glucose-induced insulin secretion (Figs. 9 and 10). In perfusion studies it was found that the second phase, but not the first phase, of glucose-induced insulin secretion was inhibited, consistent with a regulatory role for PP2B in control of β-granule transport. This implies that dephosphorylation of KHC triggers kinesin-based transport of β-granules along microtubules. Blocking KHC dephosphorylation by inhibiting PP2B prevents mobilization of β-granules and reduces the total amount of insulin available for release. Interestingly, prolonged treatment with cyclosporin A or FK506 (which are also inhibitors of PP2B (Ref. 53)) was found to inhibit glucose-induced insulin secretion (56, 57). Use of cyclosporin A/FK506 as an immunosuppressant in islet transplantation therapy for diabetes was unsuccessful, because it is detrimental for β-cell function and insulin secretion (58). This adverse effect of cyclosporin A/FK506 on insulin release is likely the result of PP2B inhibition that in turn prevents kinesin-mediated β-granule transport and lowers the β-granule pool docked at the plasma membrane available for exocytosis. However, it should be noted that inhibition of PP2B does not completely block glucose-induced insulin secretion from isolated islets, indicating that this is not the only Ca^{2+}-dependent component in insulin secretion. Other Ca^{2+}-regulated β-granule proteins are likely to play a role in Ca^{2+}-induced exocytosis from β-cells, although their identity and precise functions have yet to be defined (4).

In summary, the data in this study imply that KHC was not actively transporting β-granules in β-cells when phosphorylated by CK2 in a basal state. However, a nutrient-induced increase in β-cell cytosolic [Ca^{2+}], leads to Ca^{2+}/calmodulin-induced activation of PP2B, which dephosphorylates KHC, activating kinesin motor activity and enhancing microtubule-based β-granule transport. This PP2B-mediated Ca^{2+}/calmodulin-dependent dephosphorylation of KHC presents a first insight into a connection between generation of key secondary coupling signal (i.e., a rise in cytosolic [Ca^{2+}]) and a component of the mechanism of insulin exocytosis (i.e., increased kinesin/microtubule-based β-granule motility) (4). In addition, a previously unsuspected role for PP2B/calcinexin is revealed in control of insulin secretion.

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Ca^{2+}-dependent Dephosphorylation of Kinesin Heavy Chain on β-Granules in Pancreatic β-Cells: IMPLICATIONS FOR REGULATED β-GRANULE TRANSPORT AND INSULIN EXOCYTOSIS
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