Secretagogue-dependent phosphorylation of phogrin, an insulin granule membrane protein tyrosine phosphatase homologue

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Phogrin, a 60/64 kDa integral membrane protein localized to dense-core secretory granules of neuroendocrine cells, was found to be reversibly phosphorylated in intact pancreatic β -cells. Phosphorylation occurred in response to a variety of secretory stimuli, including glucose and depolarizing concentrations of K^+ . In MIN6 cells, the glucose dose–response and time course of phogrin phosphorylation paralleled that of insulin secretion. Like secretion, glucose- or K^+ -stimulated phosphorylation required the presence of Ca^{2+} . The calmodulin antagonist W-7 and the Ca^{2+} /calmodulin-dependent kinase II inhibitor KN-93 dose-dependently inhibited both phosphorylation and secretion, while the ‘inactive’ analogue KN-92 was effective only at significantly higher concentrations. Phosphorylation of phogrin

was also stimulated in cells exposed to forskolin, an effect presumably mediated by protein kinase A (cAMP-dependent protein kinase). Under these conditions, phogrin phosphorylation could be dissociated from the secretory response. In MIN6 cells, as in pancreatic islets, cAMP potentiates rather than initiates insulin release. Thus our observations are consistent with a role for phogrin phosphorylation in the signal-transduction pathway at a site proximal to the exocytic event itself, possibly regulating secretory-granule mobilization and recruitment to the exocytic site.

Key words: β -cell, phosphoprotein, protein kinase, secretion, signal transduction.

INTRODUCTION

The reversible phosphorylation of regulatory proteins has frequently been implicated as a key component of insulin release from dense-core granules [1,2]. Secretory stimuli activate a range of protein kinases in the pancreatic β -cell, often in a Ca^{2+} -dependent manner. Glucose, the major physiological secretagogue, acts by stimulating Ca^{2+} influx and increasing free cytosolic Ca^{2+} [3,4], an effect mimicked by direct depolarization of the cell with K^+ ions or by Ca^{2+} -ionophores. Ca^{2+} /calmodulin-dependent kinase II (CaM kinase II), the major multifunctional kinase mediating intracellular Ca^{2+} signalling [5], is rapidly activated by stimulation of isolated pancreatic islets and cultured β -cell lines with either K^+ or glucose [6–8]. A direct functional role for CaM kinase II in the secretory process, however, remains to be demonstrated. Myosin light-chain kinase (MLCK), another Ca^{2+} /calmodulin-regulated enzyme present in β -cells, has been implicated in promoting insulin release from permeabilized islets [9,10]. Activation of Ca^{2+} - and phospholipid-dependent protein kinase C (PKC) with phorbol esters stimulates insulin release; however, the fact that PKC-depleted islets retain a normal secretory response to glucose argues against an obligatory role for this enzyme [11]. Mitogen-activated protein (MAP) kinase is responsive to glucose in INS-1 insulinoma cells, but appears not to be involved directly in the secretory response as an inhibitor preventing its activation, PD98059, has no effect on hormone release [12a,12b].

A number of hormonal stimuli modulate glucose-induced insulin secretion. Glucagon and glucagon-like peptide-1, for example, are physiologically important potentiators of secretion

that increase intracellular levels of cAMP, leading to the activation of protein kinase A (cAMP-dependent protein kinase; PKA) [3,13]. While insulin release is not stimulated in the absence of glucose, PKA activity significantly potentiates glucose-induced secretion. Such cross-talk between different signalling pathways allows the β -cell to respond appropriately to a variety of physiological conditions [13].

Despite compelling evidence linking protein phosphorylation to insulin secretion, there has been little progress in identifying the molecular and cellular processes involved, and the phosphorylated substrate proteins have remained elusive. Numerous studies have demonstrated Ca^{2+} - [14–18], phorbol ester- [16] or cAMP- [14,17,19,20] dependent phosphorylation of a wide variety of β -cell proteins (see also [2]), either in intact cells, cell homogenates or subcellular fractions such as secretory granules. For the most part the substrates have not been identified, precluding more detailed functional studies. Moreover, phosphorylation events observed *in vitro* generally have not been shown to occur in intact cells. An alternative approach has been the analysis of known *in vitro* substrates as candidate proteins. Stimulus-dependent phosphorylation of microtubule-associated protein MAP-2 [8] and a synapsin I-like protein [7], substrates for CaM kinase II, or of the myristoylated alanine-rich C kinase substrate (‘MARCKS’) [21], has been observed in β -cells. It is unclear, however, whether any of these proteins are involved in modulating insulin release.

Phogrin (phosphatase homologue in granules of insulinoma) is an integral membrane glycoprotein that is widely expressed among neuroendocrine tissues, where it localizes to dense-core secretory granules [22,23]). The molecule is a member of the

Abbreviations used: CaM kinase II, Ca^{2+} /calmodulin-dependent kinase II; PKA, protein kinase A (cAMP-dependent protein kinase); MLCK, myosin light-chain kinase; PKC, protein kinase C; MAP kinase, mitogen-activated protein kinase; PTP, protein tyrosine phosphatase; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline.

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receptor-type protein tyrosine phosphatase (PTP) family, although its single cytosolic PTP domain may not be catalytically active, owing to the substitution of a highly conserved residue (Ala $\rightarrow$ Asp) within the substrate-binding pocket. Phogrin is closely related to another dense-core granule protein, IA-2 (ICA512) [24], which shares a similar domain structure and exhibits 80% amino acid sequence identity in the PTP homology region [22]. Here we report that phogrin is a phosphoprotein and demonstrate that insulin secretagogues stimulate the phosphorylation of β -cell phogrin in intact cells, making this protein a candidate for a potential regulator of insulin secretion.

MATERIALS AND METHODS

Reagents

Unless otherwise stated, reagents were of analytical grade and were purchased from Sigma Chemical Co. Forskolin, PMA, bisindolylmaleimide I, ML-7, KN-62, KN-93, KN-92 and PD98059 were obtained from Calbiochem.

Cell culture

β TC3 (mouse insulinoma) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; 25 mM glucose) (Gibco-BRL) with 10% (v/v) foetal-calf serum, 50 i.u./ml penicillin, 50 μ g/ml streptomycin and 2 mM L-glutamine at 37 °C in a humidified CO₂/air (1:19) incubator. MIN6 (mouse insulinoma) cells were cultured in the same medium supplemented with 50 μ M β -mercaptoethanol and used between passage numbers 30 and 55. Pancreatic islets were obtained from NEDH rats by collagenase digestion [25].

Western blotting

Proteins were separated by SDS/PAGE (7% gel), transferred to nitrocellulose membrane by semi-dry electrophoretic transfer, and immunoblotted using Tris-buffered saline (TBS) (pH 7.5)/0.1% Triton X-100/3% (w/v) dried milk powder for blocking and antibody incubations. Phogrin was detected with an affinity-purified rabbit polyclonal antibody raised to the luminal region of the protein [22]. Proteins were revealed with an alkaline phosphatase-conjugated secondary antibody and a proprietary fluorescent substrate (Vistra ECF reagent; Amersham) by fluor-imaging (Storm 860; Molecular Dynamics, Sunnyvale, CA, U.S.A.) and quantified using ImageQuant software (Molecular Dynamics).

Alkaline phosphatase treatment

Total cellular protein (500 μ g) was incubated with 50 units of calf intestinal alkaline phosphatase (Gibco-BRL) in the absence or presence of 100 mM β -glycerophosphate as a competing substrate for 30 min at 30 °C in 50 mM Tris (pH 8.0)/1 mM MgCl₂.

Secretagogue stimulation (bandshift assays)

Cells were plated either in 35-mm-diameter dishes or in 24-well culture plates 2 or 3 days before an experiment. They were rinsed once in PBS and pre-incubated in basal buffer [modified Krebs-Ringer buffer: 5 mM KCl/120 mM NaCl/15 mM Hepes (pH 7.4)/24 mM NaHCO₃/1 mM MgCl₂/2 mM CaCl₂/1 mg/ml ovalbumin] for 30 min (β TC3) or 2 h (MIN6) at 37 °C in a CO₂/air incubator. The cells were then incubated in the same buffer modified to include high K⁺ (55 mM KCl/70 mM NaCl), D-glucose (16.7 mM), forskolin (10 μ M) or PMA (100 nM).

Inhibitors (or DMSO as the carrier solvent) were added during the last 30 min of pre-incubation and were present throughout the stimulation period. For Ca²⁺-free buffers, CaCl₂ was replaced with 2 mM EGTA. After incubation, the cells were harvested in 1 $\times$ SDS sample buffer (containing dithiothreitol) for Western-blotting analysis.

Secretion studies

Media (1 ml/35 mm dish; 0.4 ml/16 mm well) were collected at the end of each incubation. Alternatively, 10-cm-diameter dishes of cells were incubated in 7 ml of buffer on a rocking platform in a 37 °C CO₂/air incubator and 25 μ l aliquots removed at the indicated time points. Samples were centrifuged for 5 min at 14000 g, and supernatants were analysed for insulin by radioimmunoassay using a commercial kit (Linco).

Phosphate labelling

Cells were equilibrated in basal buffer containing [³²P]P_i (New England Nuclear Corp.) (200 μ Ci/35 mm dish) for 3 h followed by incubation in basal or stimulation buffers as described above. Cells were lysed in 0.75 ml of TBS (pH 7.5)/1% Triton X-100/25 mM NaF/1 mM Na₃VO₄/10 μ M pepstatin A/10 μ M E64/1 mM PMSF. Lysates were pre-cleared by incubation with 5 mg of Cowan's strain Protein A, phogrin was immunoprecipitated with 3 μ l of anti-phogrin/luminal antiserum and antibody complexes were collected on 20 μ l of Protein A-Sepharose. Immunoprecipitates were eluted into 1 $\times$ SDS sample buffer and analysed by SDS/PAGE (7% gel) and phosphor-imaging (Storm 860; Molecular Dynamics).

Biosynthetic labelling

Cells were incubated with ³⁵S-labelled methionine/cysteine (250 μ Ci/10 cm dish) in methionine/cysteine-free DMEM for 2 h. Lysis and immunoprecipitation of phogrin were performed as described above.

RESULTS

Phogrin is a phosphoprotein

Analysis of β -cell phogrin after electrophoresis on SDS/PAGE gels by Western blotting (Figure 1, top panel, lanes 1 and 2) or by immunoprecipitation (lane 3) consistently showed two major bands of approx. 60 and 64 kDa in pancreatic islets and in cultured β -cell lines maintained in glucose-containing medium prior to harvesting. Both forms of phogrin were recognized by antisera raised either to the luminal domain of the protein (Figure 1, top panel; [22]) or to the cytosolic domain (results not shown). Alkaline phosphatase treatment of MIN6 extracts, followed by Western blotting for phogrin, resulted in the disappearance of the upper band and enhancement of the faster-migrating form (Figure 1, bottom panel). Incubation in presence of the competing phosphatase substrate β -glycerophosphate preserved the doublet, suggesting that the source of the observed size heterogeneity is phosphorylation of phogrin.

Phosphorylation is induced by secretagogues

After incubation of β -cells under non-stimulatory conditions, phogrin was predominantly in the lower-molecular-mass form. Depolarization of the cell with high concentrations of K⁺ acts as a potent secretagogue through the opening of voltage-gated Ca²⁺ channels [3]. In β TC3 or MIN6 cultured mouse β -cells, stimulation with 55 mM KCl induced a marked shift in phogrin to the

Figure 1 SDS/PAGE analysis of β -cell phogrin

Top panel: lanes 1 and 2, proteins (40 μ g/lane) from rat pancreatic islets or from MIN6 β -cells maintained in DMEM (25 mM glucose) were subjected to SDS/PAGE (7% gel) and transferred to nitrocellulose. Phogrin was detected with an affinity-purified antibody raised to the luminal domain of the protein (note: mouse phogrin migrates slightly faster than the rat molecule); lane 3, MIN6 cell proteins were biosynthetically labelled with [35 S]methionine/cysteine in DMEM (25 mM glucose) before immunoprecipitation of phogrin with an antibody to the luminal domain. Bottom panel: MIN6 cell proteins were incubated with or without alkaline phosphatase (AP) (50 units for 30 min at 30 $^{\circ}$ C), in the absence or presence of 100 mM β -glycerophosphate (GP), and analysed by Western blotting.

upper band (Figure 2, top panel). In MIN6 cells, a cell line retaining the glucose-sensitivity characteristic of pancreatic islet β -cells, a similar mobility shift was also observed after incubation with 16.7 mM D-glucose. During a 30 min exposure to either high K^{+} or glucose, up to 55% of total cellular phogrin was converted from the basal into the stimulated, higher-molecular-mass form.

Phosphate incorporation was evaluated directly by incubating MIN6 cells under basal or stimulatory conditions after pre-equilibrating the intracellular ATP pool with [32 P]P $_i$, followed by immunoprecipitation of phogrin. Stimulation with high K^{+} and glucose resulted in a single strongly labelled band. No significant incorporation of phosphate was observed under basal conditions (Figure 2, bottom panel, lanes 1 and 2). Phogrin immunoprecipitated from MIN6 cells biosynthetically labelled with [35 S]methionine/cysteine under stimulatory conditions was analysed in parallel by SDS/PAGE (lane 3). The upper of the two bands corresponded in size to the phosphate-labelled phogrin. This indicated that the protein undergoes phosphorylation

Figure 2 Phosphorylation of phogrin in secretagogue-stimulated β -cells

Top panel: MIN6 or β TC3 cells were incubated for 30 min under basal conditions (5 mM KCl/0 mM glucose), in high K^{+} (55 mM KCl/0 mM glucose) or high glucose (5 mM KCl/16.7 mM glucose). Cells were harvested and immunoblotted for phogrin; bottom panel: MIN6 cells were pre-equilibrated with [32 P]P $_i$ (200 μ Ci/35 mm dish) in basal buffer for 2.5 h and then incubated for 30 min either in basal or stimulating (55 mM KCl/16.7 mM glucose) buffer. Phogrin was immunoprecipitated with an antibody to the luminal domain of the protein. For lane 3, phogrin was immunoprecipitated from MIN6 cells biosynthetically labelled with [35 S]methionine/cysteine in DMEM (25 mM glucose) for 2 h.

Figure 3 Glucose dose-dependency of insulin secretion and phogrin phosphorylation in MIN6 cells

Cells were incubated for 2 h in modified Krebs–Ringer buffer containing the indicated concentrations of glucose, followed by Western blotting for phogrin. Media were analysed for secreted insulin. Plotted data represent the means for duplicate analyses of two separate dishes.

in a reversible manner in response to secretagogue stimulation, which would account for the presence of the higher-molecular-mass phosphoprotein *in vivo*.

Correlation of phosphorylation with insulin secretion

MIN6 cells secreted insulin in response to stimulation with glucose concentrations in the physiological range for pancreatic β -cells [26], with half-maximal release occurring at about 5.5 mM (Figure 3). The extent of phogrin phosphorylation under the

Figure 4 Time course of glucose- or K^+ -induced secretion and phogrin phosphorylation in MIN6 cells

Top left and right panels: cells were incubated in basal or stimulation buffers (top left, 55 mM KCl; top right, 16.7 mM glucose) on a rocking platform. Aliquots of media were removed after the indicated periods of time and analysed for insulin. Plotted data represent the means for duplicate analyses of samples from two independent experiments. Bottom left and right panels: cells were incubated under the same conditions as for the top left and right panels respectively and were harvested after the indicated periods of time. Samples were Western-blotted for phogrin, and upper and lower bands were quantified. Data plotted represent phogrin phosphorylated during the period of incubation as a proportion of total phogrin in each lane.

same conditions, represented by the increasing intensity of the upper band on a phogrin immunoblot, correlated well with the secretory response.

Time courses of phosphorylation were analysed over the first 20 min of stimulation with either K^+ or glucose. In both cases, phosphorylation paralleled insulin release. While exposure to depolarizing concentrations of K^+ initiated a rapid secretory response (within 30 s) (Figure 4, top left panel), insulin release in response to glucose occurred only after a lag phase of about 2–4 min (Figure 4, top right panel). This phenomenon, also

observed with pancreatic islets, is accounted for by the requirement for glucose metabolism to initiate changes in ionic movements [27,28] and is by-passed by direct K^+ depolarization. In parallel with secretion, K^+ -induced phogrin phosphorylation showed rapid kinetics (Figure 4, bottom left panel), while a lag phase of about 2–4 min was seen in the case of glucose-stimulated phosphorylation (Figure 4, bottom right panel).

Phosphorylation is dependent on Ca^{2+}

Regulated insulin secretion generally requires an increase in cytosolic Ca^{2+} , mediated by the influx of Ca^{2+} from the extracellular medium [3,4]. In Ca^{2+} -free incubation buffer containing EGTA, MIN6 cells stimulated either with K^+ or with glucose did not secrete insulin above basal levels (Figure 5, top panel). In the presence of Ca^{2+} , K^+ or glucose induced the phosphorylation of phogrin, analysed by [^{32}P]P_i labelling (Figure 5, bottom panel) or Western blotting (results not shown). The same concentrations of secretagogue in the absence of Ca^{2+} did not result in any detectable phosphate incorporation. This is consistent with the involvement of a Ca^{2+} -dependent protein kinase, either phosphorylating the molecule directly or as a component of an upstream signalling cascade.

Effects of protein kinase inhibitors on phogrin phosphorylation

The cytoplasmic domain of phogrin contains several tyrosine residues and a large number of consensus sites for different serine/threonine kinases, including protein kinase C (three), Ca^{2+} /calmodulin-dependent kinase II (seven) and PKA (seven) [29]. Phogrin was initially probed for tyrosine phosphorylation using an anti-phosphotyrosine antibody, but neither immunoprecipitation followed by Western blotting with phogrin anti-serum, nor Western blotting of immunoprecipitated phogrin, revealed any phosphotyrosine on the molecule (results not shown). Analyses using a panel of inhibitors targeting individual classes of protein kinases therefore concentrated on serine/threonine kinases, in particular those known to be dependent on Ca^{2+} .

Ca^{2+} signalling is often mediated by the Ca^{2+} -binding protein calmodulin. In MIN6 cells, the calmodulin antagonist W-7 dose-dependently inhibited phogrin phosphorylation in response to glucose, with half-maximal inhibition at 30–35 μ M (Figure 6, top panel). Insulin secretion from these cells was affected in a similar way. KN-93, an inhibitor of CaM kinase II, resulted in potent inhibition of glucose-stimulated phosphorylation (Figure 6, middle panel). This was again paralleled by an inhibition of secretion, with both responses half-maximally inhibited by 6 μ M of the drug. In contrast, the structural analogue KN-92, which is reportedly inactive as an inhibitor of CaM kinase II, showed no suppression of glucose-induced phogrin phosphorylation or secretion at a concentration of 3 μ M (Figure 6, bottom panel). It did, however, inhibit phosphorylation and insulin secretion in parallel at higher concentrations (half-maximal inhibition at 25 μ M). Such high doses of both drugs may well affect additional processes in intact cells not reflecting specific effects on CaM kinase II. A second CaM kinase II inhibitor, KN-62, also potently inhibited phosphorylation, as well as secretion (half-maximal inhibition at 2.5 μ M; results not shown). The inhibition profiles observed with W-7, KN-62 and KN-93 were consistent with an essential role for CaM kinase II in both secretagogue-stimulated phogrin phosphorylation and insulin secretion.

ML-7 (up to 10 μ M), an inhibitor of MLCK, another Ca^{2+} /calmodulin-dependent enzyme, did not suppress glucose-stimulated phogrin phosphorylation. Similarly, bisindolylmaleimide I (up to 250 nM), an inhibitor of the Ca^{2+} -dependent

Figure 5 Ca^{2+} -dependence of insulin secretion and phogrin phosphorylation

Top left and right panels: MIN6 cells were incubated for 20 min in basal or stimulation buffers (55 mM KCl or 16.7 mM glucose) containing either 2 mM $CaCl_2$ ($+Ca^{2+}$) or 2 mM EGTA ($-Ca^{2+}$). Media were analysed for secreted insulin (results shown are the means of duplicate analyses on two dishes). Bottom panel: MIN6 cells pre-equilibrated with [^{32}P]P_i (200 μ Ci/35 mm dish) under basal conditions were incubated for 30 min in basal or stimulating buffers in the presence or absence of Ca^{2+} , followed by immunoprecipitation of phogrin.

Figure 6 Effects of protein kinase inhibitors on glucose-stimulated phosphorylation and secretion

MIN6 cells were stimulated with 16.7 mM glucose for 20 min in the presence of the indicated concentrations of W-7 (top panel), KN-93 (middle and bottom panels) or KN-92 (bottom panel). Media were analysed for secreted insulin (means for duplicate analyses on three separate wells; error bars, shown where larger than the symbols, indicate S.D.), and cells were Western-blotted for phogrin. For the bottom panel, phogrin bands were quantified and results expressed as the proportion of total phogrin in the phosphorylated form. Inset: lane 1, glucose only; lane 2, +10 μM KN-93; lane 3, +10 μM KN-92.

forms of PKC, or PD98059 (up to 50 μM), which blocks MAP kinase activation, were without effect (results not shown). In all cases, identical results were obtained when the effects of kinase inhibitors on K⁺-stimulated secretion were examined (results not shown). Consistent with the inhibitor study, activation of PKC by incubation with the phorbol ester PMA did not significantly stimulate phogrin phosphorylation (C. Wasmeier and J. C. Hutton, unpublished work).

Figure 7 Forskolol-induced phosphorylation of phogrin

Top left panel: MIN6 cells pre-equilibrated with [³²P]_i were stimulated with 10 μM forskolin for 40 min, followed by immunoprecipitation of phogrin; top right panel: MIN6 cells, after pre-incubation, were stimulated with forskolin as above, and phogrin was analysed by Western blotting; bottom panel: cells were incubated under basal conditions or stimulated with either 10 μM forskolin alone, 16.7 mM glucose alone or glucose in combination with forskolin. Media were assayed for secreted insulin. Data represent the mean ± S.D. for three wells of cells.

Phogrin phosphorylation is stimulated by activators of PKA

Incubation of MIN6 cells with 10 μM forskolin, which raises intracellular cAMP concentrations by stimulating adenylate cyclase [3], led to the incorporation of [³²P]_i into phogrin, even in the absence of glucose (Figure 7, top left panel). Analysis by Western blotting showed that forskolin-stimulated phosphorylation resulted in a bandshift indistinguishable from that induced by glucose or K⁺ (Figure 7, top right panel). Unlike the glucose-mediated response, forskolin-induced phosphorylation was Ca²⁺-independent and was not inhibited by KN-93 (results not shown). While forskolin in the absence of glucose did not stimulate insulin release from MIN6 cells, it showed a marked potentiating effect on glucose-stimulated secretion, increasing it by more than twofold (Figure 7, bottom panel).

DISCUSSION

Our observation that insulin secretagogues activate the phosphorylation of β-cell phogrin represents the first demonstration of stimulus-dependent phosphorylation *in vivo* of a target located directly on the dense-core granule. Phogrin is one of the few examples of a secretory-granule membrane protein that is truly specific for this organelle, and its subcellular localization places it in a strategic position for a central role in the regulation of secretory-granule function in neuroendocrine cells.

We previously described mature phogrin as doublet bands of 60 and 64 kDa when analysed by SDS/PAGE [22]. Treatment of the protein with N-glycanase resulted in an equivalent decrease

in size of both the higher- and lower-molecular-mass forms, so that N-linked glycosylation cannot account for the presence of a doublet (C. Wasmeier and J. C. Hutton, unpublished work). In the present study we showed that the upper band results from a mobility shift induced by secretagogue stimulation of β -cells, and confirmed that it is due to the incorporation of phosphate. Under basal conditions, phogrin does not appear to be phosphorylated, or at least does not contain readily exchangeable phosphate groups. As Western blotting and immunoprecipitation analyses did not detect any phosphotyrosine (C. Wasmeier and J. C. Hutton, unpublished work), the observed phosphorylation most likely occurs on serine or threonine residues. The cytosolic domain of phogrin possesses 39 serine and 17 threonine residues, with 24 of these located in a 120-amino-acid segment between the transmembrane span and the PTP domain. Although mobility changes on a gel cannot be correlated directly with the number of residues phosphorylated, it appears that stimulation might induce phosphorylation of a series of residues. In addition to the major 60 and 64 kDa bands, one or more less prominent bands of intermediate mobility are often observed, presumably representing partially phosphorylated forms.

MIN6 cells, unlike many other β -cell lines maintained in culture, retain the glucose-responsiveness characteristic of the pancreatic islet β -cell and exhibit a robust secretory response when challenged with physiological concentrations of glucose. Glucose-induced phosphorylation of phogrin in MIN6 cells showed a similar concentration-dependency to insulin secretion. This suggests that the same biochemical mechanisms may be involved, and is consistent with phosphophogrin playing a role in stimulated secretion. Further support for such a functional relationship comes from the parallel time courses of secretion and phosphorylation in response to either K^+ or glucose and from the absolute dependence of both events on extracellular Ca^{2+} , as well as from the correlation of their inhibition profiles in the presence of protein kinase inhibitors.

The observed Ca^{2+} requirement suggests the involvement of a Ca^{2+} -dependent protein kinase in the pathway leading to phogrin phosphorylation. Inhibitor studies were consistent with CaM kinase II being involved, as both the calmodulin antagonist W-7 as well as KN-62 and KN-93, two structurally distinct inhibitors of CaM kinase II, suppressed phosphorylation in a dose-dependent manner. Pharmacological effects observed in intact cells do need to be interpreted with caution, especially since KN-62 has been shown to inhibit hormone secretion partly through an effect on the L-type Ca^{2+} channel in the plasma membrane rather than on CaM kinase II [30]. As an alternative, we used KN-93, a more recently developed compound, in conjunction with its inactive structural analogue KN-92. KN-93 proved to be a potent inhibitor of phogrin phosphorylation, whereas KN-92 was only effective at a 4–5-fold higher concentration. The parallel inhibition of secretion suggests that CaM kinase II also plays an important functional role in regulated insulin release. This is consistent with what has been proposed by others and with the finding that CaM kinase II undergoes activation in secretagogue-stimulated β -cells [6]. The downstream effectors of CaM kinase II in these cells, however, are still unknown. Multiple different isoforms of the enzyme have been described [5], many of which are expressed in β -cells [31], and it remains to be determined whether an involvement in regulated secretion is restricted to specific isoforms. A study reporting localization of CaM kinase II δ to insulin secretory granules or cytoskeletal elements [32] suggests that this subtype may be a good candidate, and it will be of particular interest to investigate its role in the phosphorylation of phogrin. It remains to be established whether CaM kinase II catalyses the reaction directly, as recombinant phogrin proved to

be a poor substrate for the enzyme *in vitro* (C. Wasmeier and J. C. Hutton, unpublished work). Rather, it may activate a downstream kinase cascade, a mechanism observed frequently in signalling pathways based on protein phosphorylation [33].

Activation of PKA, while not sufficient in itself to elicit insulin release from MIN6 cells (Figure 7) or from pancreatic islets [3,13], significantly potentiates glucose-induced secretion, and as such clearly is a potent modulator of the secretory machinery. While part of this effect appears to be due to a stimulation of Ca^{2+} influx [4], PKA largely stimulates exocytosis via mechanisms independent of the Ca^{2+} signal [34,35]. Forskolin, which raises intracellular cAMP levels, also evoked the phosphorylation of phogrin, independent of Ca^{2+} and unaffected by KN-93. The resulting bandshift was indistinguishable from that observed in glucose-stimulated cells, suggesting that Ca^{2+} -dependent as well as PKA-mediated mechanisms may converge on the phosphorylation of a common set of sites. This would be consistent with the β -cell's ability to integrate the effects of stimuli transmitted via distinct second messengers, well documented with respect to the secretory response [13]. CaM kinase II and PKA may each be able to achieve phogrin phosphorylation independently, or they may be components of the same signal-transduction pathway. In the latter case, our results would place PKA downstream of CaM kinase II. Alternatively, it is possible that Ca^{2+} effects on phogrin phosphorylation are mediated via the activation of a Ca^{2+} /calmodulin-dependent adenylate cyclase [36]; it is not known whether these enzymes are sensitive to inhibition by KN-62 or KN-93. Relationships between the different pathways remain to be determined, but an effect on phogrin of two second messengers involved in regulating secretion suggests that the protein may play a fundamental role during the secretory cycle and it is possible that the potentiating effects of forskolin or cAMP on insulin release are mediated, at least in part, via phogrin.

Which step of the granule cycle does phogrin regulate? Temporally, phogrin phosphorylation coincides with insulin release. The time resolution in this type of experiment does not, however, permit conclusions as to whether phosphorylation occurs immediately before secretion, is directly involved in exocytosis or is necessary for a subsequent step, such as the retrieval and recycling of secretory-granule membranes. Stoichiometric considerations argue against a direct role in exocytosis or in retrieval from the cell surface. Stimulation results in phosphorylation of up to 55% of total cellular phogrin over a 30 min incubation period, while glucose-dependent insulin secretion amounts to less than 5% of the total/h for MIN6 cells and about 5–10% for pancreatic islets [37]. Thus we propose that phosphorylation of phogrin may play a role at an earlier stage, such as the priming of granules for exocytosis. This could involve marking individual granules as sufficiently mature for secretion, or may facilitate their movement from the cell interior to the periphery. Stimulation of granule movement in response to secretagogues has been observed in living β -cells using video imaging, either by phase-contrast microscopy [38] or with green-fluorescent-protein-labelled phogrin as a granule marker [23]. A study examining the effects of a range of secretagogues on HIT T15 β -cells demonstrated significant stimulation of granule movement by both glucose and forskolin, but not by PMA. Exposure of the cells to intracellular Ca^{2+} -chelators or to W-7 suppressed granule motility, suggesting that a Ca^{2+} /calmodulin-dependent protein kinase may be involved [38]. Capacitance measurements in single islet β -cells showed transient exocytosis in response to a rise in intracellular Ca^{2+} alone, while sustained secretion was observed when cAMP was present in addition. These data suggest the initial rapid release of a small number of primed

granules (an estimated 35–80/cell), with a sustained secretory response being dependent on refilling of this readily releasable pool [39]. The stimulation by cAMP and sensitivity to PKA inhibitors argue for a key role of PKA in mobilization and priming of granules. Such observations are consistent with our hypothesis that phogrin, activated by phosphorylation, is involved in regulating intracellular granule movement. This is compatible with the finding that forskolin, which significantly potentiates, but does not initiate, insulin secretion in islets or MIN6 cells, stimulated phogrin phosphorylation in the absence of a secretory response. Reversible phosphorylation-dependent interaction with cytoskeletal elements and associated proteins, such as microtubules, which have been implicated previously in the trafficking of secretory granules [40], may form the molecular basis for such a mechanism. In a cell like the pancreatic β -cell, which is able to sustain hormone secretion over an extended period of time, transport of granules to the site of exocytosis is an important component of the secretory response and is a step likely to be subject to secretagogue-dependent regulation. More direct experiments will be required to establish which residues in phogrin are phosphorylated and whether these sites are essential to the proposed involvement of phogrin in the secretory process.

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