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Protein phosphorylation in the pancreatic B-cell

by D.E. Harrison, S.J.H. Ashcroft, M.R. Christie and J.M. Lord

Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Headington, Oxford OX3 9DU (Great Britain)

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1. *Introduction*

The well-established modulatory role of cyclic AMP in insulin secretion (for review see Sharp[51]) affords presumptive evidence that protein phosphorylation mechanisms influence the release process. In view of the dependence of insulin secretion on Ca^{2+} and the growing awareness of the importance of Ca^{2+}-dependent protein kinases in regulating cellular activities, it was hypothesized[2] that protein phosphorylation might be the common mode of action of both Ca^{2+} and cyclic AMP in controlling insulin release. Demonstration of the presence of calmodulin in islets[18,56] supported the hypothesis that Ca^{2+}-calmodulin-dependent protein kinases are regulatory enzymes in the exocytotic release process. The discovery of Ca^{2+}- and phospholipid-dependent protein kinase and elucidation of the role of diacylglycerol in the regulation of its activity (for review see Nishizuka[38,39]) suggested that the enhanced inositol phospholipid breakdown seen in stimulated islets of Langerhans (for review see Best and Malaisse[4]) might also be linked to protein phosphorylation.

In this review we summarize the progress that has been made in characterizing islet protein kinases regulated by cyclic AMP, Ca^{2+} or diacylglycerol, and the as yet fragmentary data on the presence, localization and possible role of intracellular protein substrates for these kinases. We have therefore restricted ourselves to those enzymes which may be presumed to be involved in the control of insulin secretion. While it is possible that other cellular processes within the B-cell e.g. insulin biosynthesis are also regulated by phosphorylation–dephosphorylation, to date no studies have been performed in this area. Possible candidates for study in this context would be the casein kinases which have been demonstrated to be highly active in islets[55] but whose nature and mode of regulation are unknown.

In order to implicate protein phosphorylation as a regulatory mechanism in a cellular process such as secretion, the following criteria (adapted from Walsh and Cooper[60]) must be satisfied:

1. The presence of the particular protein kinase in the cells must be shown.
2. The cells must be demonstrated to contain endogenous substrate(s) for the kinase; and phosphatase(s) must be shown to be present to reverse the effect of the kinase.
3. In the intact cell it must be possible to demonstrate changes in extent of phosphorylation of such endogenous substrate(s) on stimulation of secretion.
4. The endogenous substrate(s) must be shown to bear a functional relationship to secretion; and changes in extent of phosphorylation must modify the activity of the protein substrate(s) in the secretory process.

For insulin secretion from the pancreatic B-cell, considerable progress has been made in demonstrating and characterizing various protein kinases in islets or B-cells, and in confirming the presence of endogenous protein substrates; protein phosphatase activity has been little studied, however. Thus the first two criteria have been at least partially met. As far as the implication of specific phosphoproteins in secretion is concerned, it has been possible in a few studies to demonstrate changes in phosphorylation occurring in conjunction with insulin secretion, but the nature of such proteins and their role (if any) in secretion has not been elucidated. Fulfilment of criteria 3 and 4 remains an elusive goal. The major protein kinase activities that have been studied in islets of Langerhans are listed in table 1.

2. *Methodological considerations*

We shall not present here detailed protocols for study of protein phosphorylation in subcellular fractions or intact insulin-secreting tissues; these can be found in the

Table 1. Protein kinases in B-cells

Protein kinase (PrK)	Substrate	References
Cyclic AMP-dependent PrK	Histone HIIA	35, 55
Casein kinase	Casein	55
Myosin light chain kinase	Myosin light chain	21, 32, 41
Ca^{2+}-calmodulin-dependent PrK	Endogenous protein(s)	10, 20, 29, 33, 47
Ca^{2+}-phospholipid-dependent PrK	Histone HI	31, 59

The table lists those protein kinase activities which have been characterized in extracts of insulin-secreting tissues. The substrates given are those that have been used for assay of the kinase.

references cited. However, certain practical problems occur in the design and interpretation of experiments of this nature and these will be briefly discussed.

a) Protein phosphorylation in subcellular systems

These studies are performed by following the incorporation of [^{32}P] from [γ-^{32}P]ATP into exogenous or endogenous protein in the presence of tissue extracts. Two technical points require especial attention. First, crude extracts of islets contain very high ATPase activity which rapidly degrades the added ATP, so that reaction rates are linear for only a short period. The time course must be carefully established to ensure that true initial velocities are being measured. Secondly, the presence of proteolytic activity in crude extracts can lead to alteration or abolition of kinase activity or appearance of artefactual bands of radioactive protein substrates. It is strongly recommended that a range of protease inhibitors be added to reaction media containing crude tissue extracts.

b) Protein phosphorylation in intact islets

These studies are carried out by pre-incubating intact islets with $^{32}P_i$ to label intracellular ATP. Following addition of a suitable stimulus, tissue is extracted and phosphoproteins analysed after various times of incubation. At isotopic equilibrium the specific radioactivity of the γ-phosphate of intracellular ATP will equal that of the P_i. However prior to attainment of equilibrium the possibility arises of artefactual results if the specific radioactivity of ATP changes on addition of the stimulus, through alteration in the rate of P_i uptake, ATP turnover or both. For example, glucose has been shown to affect markedly the rate of equilibration of ATP[9]. Direct measurement of the specific radioactivity of the γ-phosphate of intracellular ATP should be carried out under the experimental conditions used, in order to control this source of error. Even under optimal conditions, a preincubation period of 3 h is required to attain isotopic equilibrium in rat islets[9].

3. Cyclic AMP-dependent protein kinase (cAMP-PrK)

Cyclic AMP-Prk activity in islet homogenates is measured using exogenous histone as substrate. Montague and Howell[35] showed that 85% of cAMP-PrK activity in guinea pig islets is in the post-microsomal supernatant. Following $(NH_4)_2SO_4$ precipitation of the supernatant fraction the activity was eluted as a single peak from DEAE-cellulose. The partially purified preparation when subjected to gel filtration on Sephadex-G200 emerged as a single peak corresponding to a mol.wt of 180,000. Treatment of the protein with cyclic AMP before gel filtration caused dissociation of the cyclic AMP-binding activity from the protein kinase activity: the latter was eluted in a position corresponding to a mol.wt of 75,000 and the former of 90,000. No evidence was obtained in this study for different molecular forms of cAMP-PrK. However Sugden et al.[55], using DEAE-cellulose chromatography demonstrated that rat islets contain two isoenzymes of cAMP-PrK corresponding to 'Type I' and 'Type II' cAMP-PrK holoenzymes found in other tissues. The two isoenzymes of cAMP-PrK differ in their ease of dissociation by preincubation with histone or NaCl. As in other tissues they are likely to share the same catalytic subunit. A mol.wt of 144,200 was calculated for rat islet cAMP-PrK from measurement of sedimentation coefficients and Stokes' radius. The reported physical and kinetic properties of islet cAMP-PrK are summarized in table 2.

We have shown previously that endogenous cAMP-PrK in rat islet homogenates phosphorylates polypeptides of M_r 55 and 70–80 kDa[20]. It was speculated that the 55 kDa polypeptide may be the same as the major species phosphorylated by a Ca^{2+}-calmodulin-dependent protein kinase (see next section). However, it has recently been shown that these are distinct polypeptides and that this substrate for cAMP-PrK may be the regulatory subunit of the Type II enzyme[28].

A limitation of these studies is the relatively small activity of endogenous cAMP-PrK in islet homogenates compared with cAMP-independent protein kinases[55]. We have recently used exogenous purified catalytic subunit of cAMP-PrK to demonstrate islet substrates for this enzyme (M.R. Christie, unpublished observations). After separation of nuclei and intact cells by low-speed centrifugation, rat islet homogenates were centrifuged at 190,000 × g for 30 min to prepare a particulate and a soluble fraction. Fractions were incubated with [γ-^{32}P]ATP in the presence or absence of purified catalytic subunit. As shown in figure 1(a), the major species phosphorylated by catalytic subunit had M_r's of 90, 60, 32, 23, 17 and 16 kDa in the particulate fraction and 60, 57, 30, 25 and 18 kDa in the soluble fraction. The 57-kDa protein may correspond to regulatory subunit, as discussed above. The 32-kDa particu-

Table 2. Properties of cyclic AMP-dependent protein kinase in rat islets of Langerhans

Activity ratio (Type I)	0.17
(Type II)	0.38
K_m for histone HIIA	0.08 mg/ml
K_m for cyclic AMP	0.08 μM
K_m for ATP (Type I)	16.1 μM
(Type II)	15.4 μM
Mol.wt	144,200
Sedimentation coefficient	6.7 S
Stokes' radius	5.2 nm
Frictional ratio	1.5

Data are from Sugden et al.[55]. Activity ratio is the ratio of the activity in the absence of cyclic AMP to that in its presence. Mol.wt and frictional ratio were calculated from the sedimentation coefficient and Stokes' radius.

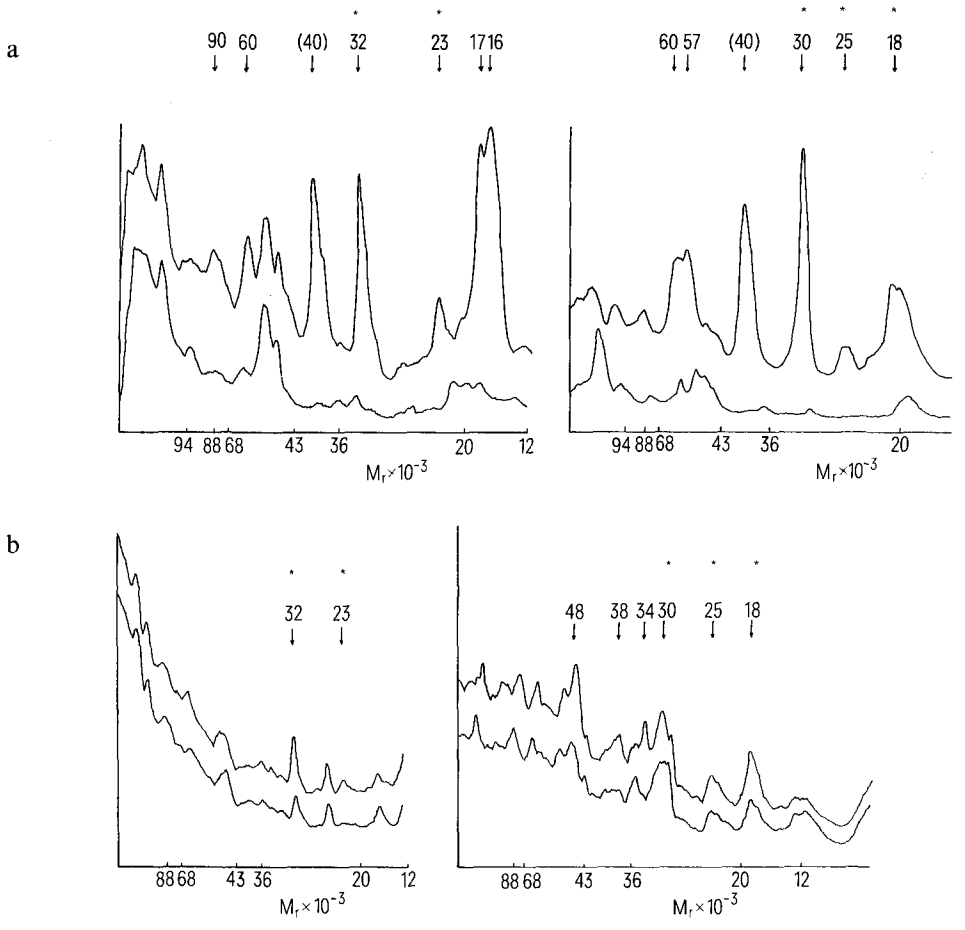

Figure 1. Endogenous substrates for cyclic AMP-dependent protein kinase in rat islets of Langerhans. *a* Homogenates of rat islets were centrifuged at 600 × g for 5 min to sediment nuclei and cell debris. The low speed supernatant was centrifuged at 190,000 × g for 30 min to prepare a particulate and a soluble fraction. The fractions were incubated with [γ-^{32}P]ATP in the absence (lower traces) or presence (upper traces) of exogenous catalytic subunit of cyclic AMP-dependent protein kinase and then subjected to electrophoresis on SDS-polyacrylamide gels. The figure shows densitometric traces of the autoradiographs from the particulate (left panel) and soluble (right panel) fractions. The arrows mark the positions of the major bands whose phosphorylation was enhanced by catalytic subunit and their M_r values ($\times 10^{-3}$) are indicated above the arrows. The 40-kDa band seen in both panels is not an endogenous islet protein but a phosphorylated component of the preparation of catalytic subunit used. *b* Islets were incubated for 3 h in Hepes-buffered bicarbonate containing $^{32}P_i$ in the absence (lower traces) or presence (upper traces) of 0.1 mM IBMX. After incubation islets were washed, fractionated and electrophoresed as described above. The figure shows densitometric traces of the autoradiographs from the particulate (left panel) and soluble (right panel) fractions. The major bands whose phosphorylation was enhanced by IBMX are indicated by arrows and their M_r values ($\times 10^{-3}$) are given above the arrows.
The bands showing enhanced phosphorylation in both systems are indicated by asterisks.

late protein is likely to be ribosomal protein S6[50]. Studies in intact islets (see below) show that some, but not all, of the peptides become phosphorylated in response to elevation of islet cyclic AMP.

An early approach to implicating cAMP-PrK in the control of insulin release was that of Montague and Howell[36] who demonstrated that the activity of cAMP-PrK in islet extracts was greater if the islets had been incubated with agents that increased islet cyclic AMP content and insulin secretion; the increased activity was ascribed to dissociation of the holoenzyme to liberate free catalytic subunit. Schubart et al.[50] studied cyclic AMP-dependent protein phosphorylation in a transplantable hamster islet cell tumor. Only one ^{32}P-labelled protein band displayed a significant increase when intact cells, pre-labelled with $^{32}P_i$, were incubated with glucagon or 8-bromo-cyclic AMP. The phosphoprotein had a M_r on SDS-gel electrophoresis of 28,000 and was identified as ribosomal protein S6: whatever the function of this phosphorylation it is unlikely to be involved in secretion. The same group[46] using 2-dimensional electrophoresis, have found that glucagon enhances phosphorylation in intact insulinoma cells of two peptides of M_r 16,000, both minor cytosolic components. In rat islets, Suzuki et al.[58] reported that glucagon or dibutyryl cyclic AMP enhanced the phosphorylation of 15 polypeptides. The effects were detected as early as 2 min after stimulation; the increased phosphorylation elicited by glucagon, but not dibutyryl cyclic AMP, was blocked by somatostatin. The subcellular localization or identification of the phosphopeptides was not reported. Christie and Ashcroft[9] compared the effects on insulin release, cyclic AMP content and protein phosphorylation in rat islets of Langerhans of glucagon, dibutyryl cyclic AMP, cholera toxin and 3-isobutyl-1-methylxanthine (IBMX). In response to an increase in cyclic AMP, the major effect on protein phosphorylation was on a protein of M_r 15,000 on SDS-gels. The extent of

phosphorylation of the M_r-15,000 protein was correlated with the level of cyclic AMP: phosphorylation in response to IBMX was inhibited by 2-deoxyadenosine, an inhibitor of adenylcyclase. Fractionation of islets, however, suggested that the M_r-15,000 protein was of nuclear origin: acetic acid/urea/Triton gel electrophoresis identified the protein as Histone H3. This phosphorylation is unlikely to be related to exocytosis, but could be relevant to effects of cyclic AMP on cell division. After removal of nuclei by centrifugation at 600 × g, the islets were further fractionated by centrifugation at 190,000 × g for 30 min. This revealed that IBMX increased phosphorylation of soluble proteins of M_r 48, 38, 34, 30, 25 and 18 kDa. Recent studies (M. R. Christie, unpublished observations) have compared the pattern of protein phosphorylation in response to elevation of cyclic AMP in the intact islet by IBMX with that obtained with islet extracts and gexogenous catalytic subunit described above. Figure 1(b) shows that proteins of M_r 32 and 23 kDa in the particulate fraction and of 30, 25 and 18 kDa in the supernatant were substrates in both systems.

4. *Ca^{2+}-calmodulin-dependent protein kinases*

Calmodulin has been shown to occur in rat pancreatic islets[18, 56] and in rat insulinoma[24]. The high degree of structural conservation observed between calmodulins from various sources suggests that it plays a fundamental role in mediating Ca^{2+}-dependent cellular events. The most important features of calmodulin are its ability to bind Ca^{2+} with dissociation constants (K_d) in the range 10^{-5} to 10^{-6} M and the significant conformational changes which occur as a result of Ca^{2+} binding. Presumably, it is the change in conformation which allows the Ca^{2+}-calmodulin complex to bind to calmodulin-dependent enzymes and thus to activate them. The physiological significance of the K_d values for Ca^{2+} binding to calmodulin becomes apparent upon examination of the changes in free Ca^{2+} concentration within the cytosol of cells during stimulus-response coupling events. In unstimulated cells the cytosolic free Ca^{2+} concentration is of the order 10^{-8} to 10^{-7} M; upon stimulation this value has been found to rise to 10^{-5} M. We envisage that Ca^{2+} may act as a second messenger through calmodulin by activating specific protein kinases.

Several Ca^{2+}-calmodulin-dependent protein kinase activites have been identified in extracts of insulin containing tissue. Using exogenous substrates it has been possible to identify two known Ca^{2+}-calmodulin-dependent protein kinases – namely myosin light chain kinase[21, 32, 41] and phosphorylase kinase (D. E. Harrison, unpublished observations). Of these two enzymes, the former has more attractive possibilities as a component of the secretory system and thus merits further discussion.

Myosin light chain kinase (MLCK) has been partially purified by ion exchange chromatography and calmodulin affinity chromatography from a rat insulinoma[41] and from rat islets[32]. Phosphorylation of exogenous myosin light chains was stimulated by micromolar concentrations of Ca^{2+} in the presence of calmodulin; trifluoperazine inhibited light chain phosphorylation. We have also been able to demonstrate MLCK in human islets (D. E. Harrison, unpublished observations). Figure 2 shows phosphorylation of exogenous myosin light chains catalyzed by an extract of human islets: phosphorylation of myosin light chains seen in the presence of Ca^{2+} (channel 5) was stimulated by addition of calmodulin (channel 6); this stimulation was reduced by trifluoperazine (channel 7).

A problem particularly in the case of the insulinoma, yet also to a lesser extent with the islet as well, is the possibility that the MLCK is derived from vascular tissue associated with the insulin-containing tissue. Using extracts of a cloned B-cell line (HIT T15) which can be grown in tissue culture (and thus is not contaminated with other cell types), we have been able to demonstrate that the phosphorylation of exogenous myosin light chains seen in the presence of Ca^{2+} and calmodulin is reduced by 50–60% upon removal of Ca^{2+} or addition of trifluoperazine (D. E. Harrison, unpublished observations). This proves unequivocally the presence of this enzyme in B-cells.

In smooth muscle and other nonmuscle cells, phosphorylation of myosin is a prerequisite for actomyosin ATPase activation and contraction. It is tempting to speculate that phosphorylation of myosin in the B-cell facilitates interaction of actin and myosin and that the resultant ATPase activation provides the motile force for movement of the insulin secretory granules to the pe-

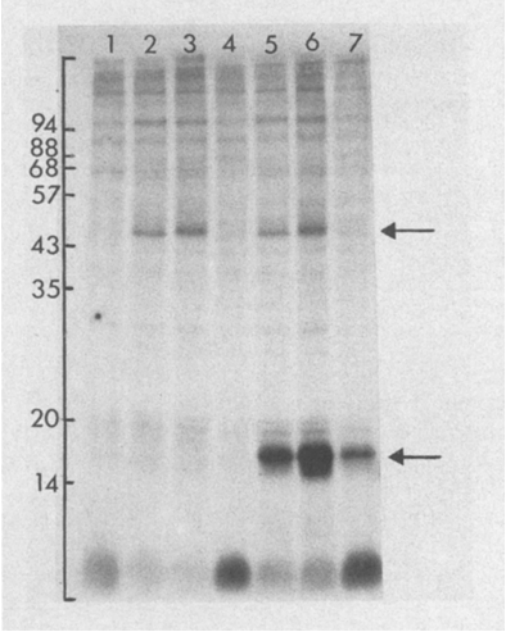

Figure 2. Protein phosphorylation by extracts of human islets. Islet extracts were incubated for 1 min with 33 μM [γ-^{32}P]ATP and 1 mM $MgCl_2$ in the presence of 1 mM EGTA (channel 1) or 10 μM Ca^{2+} (channels 2–7). Additions to incubations were as follows: 1.25 μM calmodulin (channels 1, 3, 4, 6, 7), 100 μM trifluoperazine (channels 4 and 7) and 2 μg myosin light chains (channels 5, 6, 7). Phosphorylated proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. The positions of the 48-kDa endogenous polypeptide substrate for Ca^{2+}-calmodulin-dependent protein kinase and exogenous myosin light chains are indicated by the arrows upper and lower arrows respectively. The numbers on the left hand side of the photograph indicate the positions of protein standards ($M_r \times 10^{-3}$).

riphery of the cell. There is evidence[23] that insulin secretory granules interact with actomyosin as do also chromaffin granules[7].

Islets also contain a Ca^{2+}-calmodulin-dependent protein kinase whose major endogenous substrate is a polypeptide whose mol.wt has variously been reported to be in the range of 53–57 kDa[10, 20, 29, 33]. We have characterized this enzyme in homogenates of rat islets of Langerhans[20] and have found the enzyme phosphorylates a 53-kDa polypeptide (P53). Maximal phosphorylation of this protein occurred in the presence of 2 μM free Ca^{2+} and 0.7 μM calmodulin. Incorporation of label into P53 was inhibited by the calmodulin antagonists trifluoperazine (TFP) and W7 but not by cyclic AMP-dependent protein kinase inhibitor. The effects of Ca^{2+}, calmodulin and these inhibitors on phosphorylation of P53 by rat islet homogenates are summarized in figure 3. Similar results have been reported by Landt et al.[29] using rat pancreatic islet cell membranes.

The nature, and even cellular location, of the endogenous substrate is unresolved. In their studies, Landt et al.[29] observed a 57-kDa substrate in islet microsome fractions. Further studies by the same workers[10] resolved the substrate into two phosphorylated polypeptides of M_r 54 kDa and 57 kDa; several lines of evidence were presented which suggested that the identity of these two polypeptides may be the α- and β-subunits of tubulin. On the other hand, MacDonald et al.[33] describe the occurrence of a 57-kDa substrate in the soluble protein fraction – this was originally suggested to be pyruvate kinase. Curiously, in a more recent paper from the same group[28] this possibility is not even mentioned. Brocklehurst and Hutton[5] also observe a 57-kDa cytosolic substrate in rat insulinoma. No studies on the identity of this protein have been described.

In our own studies (D. E. Harrison, unpublished observations), we have found the 53-kDa substrate to occur predominantly in the particulate fraction after sonication and centrifugation of rat islet extracts. This can be seen in figure 4: phosphorylation of P53 in the particulate fraction was totally dependent on the presence of both Ca^{2+} and calmodulin (channel C2); Ca^{2+} alone had no effect on phosphorylation in this fraction (channel C1) suggesting that all endogenous calmodulin had been removed by centrifugation. In the supernatant fraction, some phosphorylation of P53 was seen in the presence of Ca^{2+} alone (channel B1); in this case, addition of calmodulin had only a modest stimulatory effect (channel B2). We have also found that the kinase and its substrate are closely associated (raising the possibility of autophosphorylation) and attempts to separate the two have been unsuccessful. If precautions are taken to prevent proteolysis, P53 is insoluble in high or low ionic strength media and in nonionic detergents such as Triton X-100. These properties are similar to those of cytoskeletal proteins and we have observed phosphorylation of P53 in islet cytoskeletons. Of the cytoskeletal proteins, likely candidates would include intermediate filament protein or membrane bound tubulin. The latter would correlate with the observations of Colca et al.[10], while in intact hamster insulinoma cells increased phosphorylation of an intermediate filament protein, possibly vimentin, has been observed upon stimulation of insulin release by depolarization dependent Ca^{2+} influx[46]. In our own studies we have been able to demonstrate phosphorylation of rat brain tubulin by islet extracts; however we have not observed any stimulation of this phosphorylation by Ca^{2+} and calmodulin. Evidence

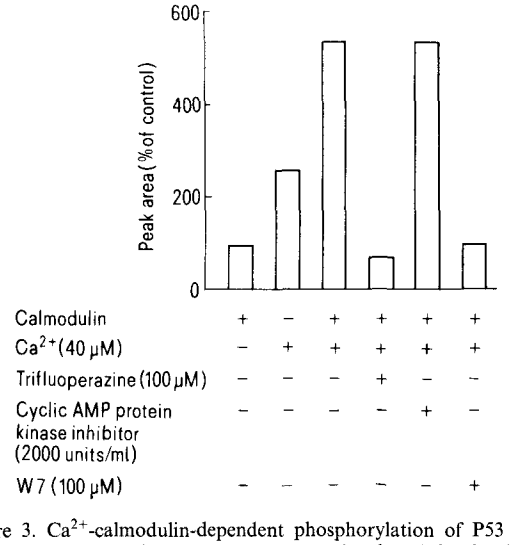

Figure 3. Ca^{2+}-calmodulin-dependent phosphorylation of P53 by rat islet homogenates. Islet homogenates were incubated for 2 min with [γ-^{32}P]ATP and the additions shown below the histogram. Phosphorylated proteins were separated by SDS polyacrylamide gel electrophoresis and detected by autoradiography. Phosphorylation of P53 was estimated by densitometry and is expressed as percent of control incubations in the absence of any addition.

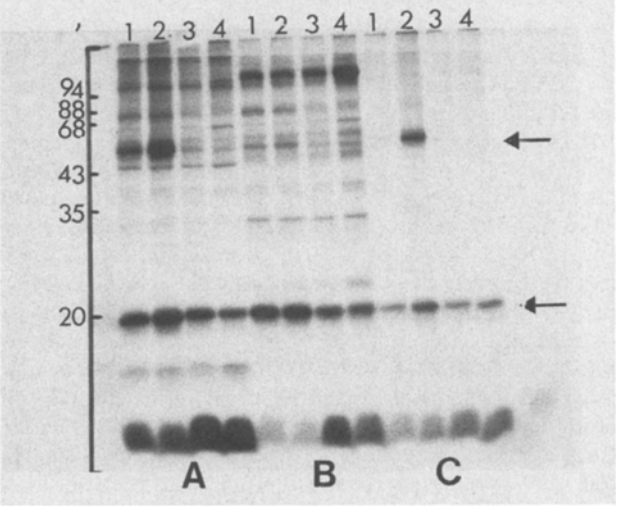

Figure 4. Distribution of P53 in subcellular fractions of rat islets. Rat islets were sonicated in 40 mM imidazole pH 7.0 containing 0.6 M KCl, 1 mM DTT and proteolytic inhibitors. After centrifugation for 1 h at 190,000 × g fractions were incubated for 1 min in the presence of 33 μM [γ-^{32}P]ATP and 1 mM $MgCl_2$. Where appropriate, incubation media contained Ca^{2+} (50 μM), calmodulin (1.25 μM), trifluoperazine (100 μM), EGTA (1 mM) and myosin light chains (2 μg). Phosphorylated proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. The autoradiograph shows the effect of Ca^{2+} (1); Ca^{2+} and calmodulin (2); Ca^{2+}, calmodulin and TFP (3) or calmodulin and EGTA (4) on phosphorylation catalyzed by the homogenate (A), supernatant (B) or pellet (C) fractions. The upper and lower arrows indicate the position of P53 and added myosin light chains respectively. The numbers on the left hand side of the photograph indicate the positions of protein standards ($M_r \times 10^{-3}$).

from other tissues suggest[5] that either protein may be a candidate for P53. Ca^{2+}-calmodulin-dependent protein kinases have been identified which phosphorylate either synaptic vesicle tubulin[6] or Sertoli cell intermediate filament protein[54].

Most studies on insulin secretion have depended on use of islets from experimental animals yet it is important to show that such models are applicable to man. It is therefore significant that extracts of human islets contain Ca^{2+}-calmodulin-dependent protein kinases. In addition to myosin light chain kinase, figure 2 shows that the major endogenous substrate for Ca^{2+}-calmodulin-dependent protein kinase in human islets is a polypeptide of molecular weight 48 kDa. Phosphorylation of this polypeptide is stimulated by Ca^{2+} (channel 2) or Ca^{2+} plus calmodulin (channel 3); removal of Ca^{2+} (channel 1) or addition of trifluoperazine (channel 4) abolishes this stimulation. These data provide for the first time an enzymic basis for involvement of protein phosphorylation in stimulus-secretion coupling in the human as well as the rodent islet.

While the exact nature and number of protein substrate(s) remains in doubt, it is significant that the presence of the Ca^{2+}-calmodulin-dependent protein kinase and its endogenous substrate have been reported by several laboratories and in several types of B-cell containing tissue. Furthermore, a similar enzyme has been reported in other secretory tissues including lacrimal gland[12], exocrine pancreas[19] and nervous tissue[14]. This suggests that this enzyme may be central to the exocytotic release mechanism and it is therefore important to establish the identity of the protein substrate and the effect of phosphorylation upon it.

Demonstration of changes in protein phosphorylation in intact stimulated islets related to changes in activity of calmodulin-dependent enzymes has been rather unsuccessful. Although islets contain both myosin[40] and MLCK there are no reports of changes in the phosphorylation state of the 20-kDa light chain upon stimulation. Colca et al.[10] claim to have demonstrated changes in phosphorylation of the smaller endogenous substrate (54 kDa) of the Ca^{2+}-calmodulin protein kinase. However, the changes in phosphorylation of this protein in response to glucose were somewhat modest (see section 6). Schubart[46,48,49] has reported changes in phosphorylation of a 60-kDa protein when insulin release from hamster insulinoma was induced by depolarization-dependent Ca^{2+} influx. Although the major Ca^{2+}-calmodulin-dependent phosphorylation seen in cell-free extracts of the insulinoma was of a 98-kDa protein[47], it is possible that the 60-kDa protein, which was tentatively identified as vimentin[46], is similar in nature to P53 found in normal islets.

A problem in ascribing effects on secretion to changes in activity of Ca^{2+}-calmodulin-dependent protein kinases is that no specific inhibitors or activators of the enzyme are available. Drugs such as TFP and W7 which have been used to implicate calmodulin in insulin release[18] do not discriminate between Ca^{2+}-calmodulin-dependent protein kinase and Ca^{2+}-phospholipid-dependent protein kinase[44,45]. Possibly the most convincing evidence to date suggesting involvement of Ca^{2+}-calmodulin-dependent protein kinase in insulin release is the demonstration that pretreatment of intact islets with concentrations of alloxan which inhibit insulin release similarly inhibit this protein kinase activity[11]. As with insulin release, glucose was shown to protect against the inhibitory effects of this diabetogenic compound. Alloxan also directly inhibited the enzyme when assayed in homogenates. That this may be a specific effect of alloxan was suggested by the fact that no significant alteration of phosphorylation of other protein bands was observed in the presence of alloxan. Thus, alloxan may prove to be a useful tool in elucidating the role of Ca^{2+}-calmodulin-dependent protein kinase in insulin secretion.

5. Ca^{2+}- and phospholipid-dependent protein kinase (Ca-PL-PrK)

Ca-PL-PrK is a Ca^{2+}-dependent protein kinase that requires the presence of an acidic phospholipid e.g. phosphatidylserine for activity and whose sensitivity to Ca^{2+} is markedly enhanced by the presence of diacylglycerol (for review see Nishizuka[38,39]). Since an increase in concentration of the latter is a consequence of enhanced inositol lipid turnover, it is possible that diacyglycerol is the intracellular mesenger for secretagogues which provoke the PI response in islets.

Ca-PL-PrK has been detected in extracts of rat islets using histone H1 as substrate[31,59]; the enzyme has been highly purified from cloned hamster B-cells[31]. Physical and kinetic properties of the purified B-cell Ca-PL-PrK are summarized in table 3. The dependence of the activity on Ca^{2+} concentration is shown in figure 5. The subcellular location of the enzyme is dependent on Ca^{2+}. When rat islets are homogenized in the absence of Ca^{2+} and presence of EGTA, Ca-PL-PrK is predominantly located in a 190,000 × g supernatant fraction. In the presence of Ca^{2+}, the majority of Ca-PL-PrK activity is found in the particulate fraction (J.M. Lord, unpublished observations).

Endogenous protein substrates for Ca-PL-PrK can be demonstrated in extracts of islets or B-cells in the absence of exogenous histone. Figure 6 shows traces of

Table 3. Properties of Ca^{2+}-phospholipid-dependent protein kinase purified from cloned hamster B-cells (HIT-T15)

Substrate or effector	Activity ratio	Concentration giving half maximum rate
ATP	–	10 μM
Histone HI	–	0.5 μM
Ca^{2+}	0.25	3.9 μM
Phosphatidylserine	0.20	18 μg/ml
Diolein	0.26	2.5 μg/ml
Mol. wt		85,200
Sedimentation coefficient		4.7 S
Stokes' radius		4.4 nm
Frictional ratio		1.5

Data are taken from Lord and Ashcroft[31] and were obtained on a highly purified enzyme from virally-transformed hamster B-cells (HIT-T15). Activity ratios are the ratio of reaction velocity in the absence of effector to that in the presence of a maximally stimulating concentration of effector when the concentration of the other two effectors were constant but not saturating viz 1 μM Ca^{2+}, 16 μg/ml phosphatidylserine and 1.2 μg/ml diolein. Mol. wt was calculated from sedimentation coefficient and Stokes' radius: a similar value was obtained from SDS-polyacrylamide gel electrophoresis.

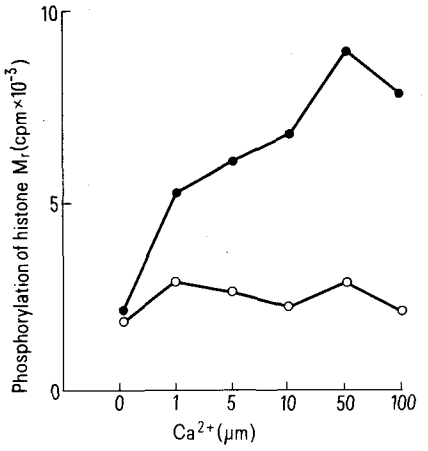

Figure 5. Stimulation of B-cell Ca^{2+}-phospholipid-dependent protein kinase by Ca^{2+}. The activity of Ca-PL-PrK purified from HIT-T15 cells was measured as described in Christie and Ashcroft[9] in the presence of Ca^{2+} at the concentrations shown and in the absence (○) or presence (●) of 16 μg/ml phosphatidylserine and 1.2 μg/ml diolein. Reactions were for 5 min at 30 °C.

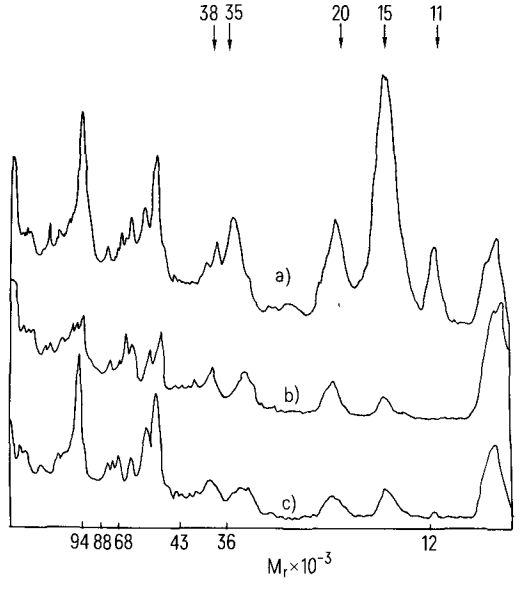

Figure 6. Phosphorylation of endogenous protein substrates by Ca^{2+}-phospholipid-dependent protein kinase in extracts of rat islets. A 40,000 × g supernatant fraction from rat islet homogenate prepared as in Christie and Ashcroft[9] was incubated with [γ-^{32}P]ATP and the following additions: *a* 50 μM Ca^{2+}, 16 μg/ml phosphatidylserine, 1.2 μg/ml diolein; *b* 10 mM EGTA, 16 μg/ml phosphatidylserine, 1.2 μg/ml diolein; *c* 50 μM Ca^{2+}. Phosphopeptides were separated by electrophoresis on SDS-polyacrylamide gels. The figure shows densitometric traces of the autoradiographs. The bands whose phosphorylation was enhanced by Ca^{2+} plus phosphatidylserine and diolein are indicated by arrows and their M_r values (× 10^{-3}) are given above the arrows.

autoradiographs of SDS-gels of islet extracts incubated with [γ-^{32}P]ATP. The peptides whose phosphorylation is enhanced by Ca^{2+} plus phospholipid have M_r's of 38, 35, 20, 15 and 11 kDa; their nature is so far unknown. Brocklehurst and Hutton[5] have studied protein phosphorylation in a granule fraction purified from a rat islet-cell tumor. Granules alone did not show Ca^{2+}-dependent protein phosphorylaton; however, when the granules were incubated with a soluble protein fraction, Ca^{2+}-dependent phosphorylation of proteins of M_r 10,000, 29,000 and 100,000 was observed. These phosphorylations were not enhanced by exogenous calmodulin nor abolished by removal of endogenous calmodulin from the soluble fraction; they were inhibited by trifluoperazine however. The authors suggested that the activity could therefore be attributed to Ca-PL-PrK.

In intact cells, several approaches have been used to implicate Ca-PL-PrK in regulation of insulin secretion. Tanigawa et al.[59] incubated rat islets of Langerhans with phospholipase C and found a 20-fold increase in insulin secretion. The data were interpreted in terms of liberation of intracellular diacylglycerol and consequent stimulation of Ca-PL-PrK. The view that phospholipase was inducing phospholipid breakdown was supported by the observed changes in phospholipid turnover; however, no data were given to demonstrate that Ca-PL-PrK had been activated.

A number of agents modify B-cell Ca-PL-PrK in vitro and may be of use in implicating the enzyme in the secretory process; the enzyme is markedly stimulated by the tumor-promoting phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) and is inhibited by trifluoperazine or by vitamin A (table 4). As also shown in table 4, these agents similarly affect insulin secretion and thus the results are at least consistent with the involvement of the enzyme in insulin release. However, both trifluoperazine and vitamin A have known effects on other parameters of islet function viz. calmodulin[18, 56] and glucose oxidation[8] respectively, and caution must be exercised in interpreting these data. Chloropromazine and dibucaine also inhibited both Ca-PL-PrK and insulin release[59] but again do not distinguish between effects on Ca-PL-PrK or calmodulin.

The characteristics of TPA-stimulated insulin release have been studied in some detail. Marked synergism has been found between TPA and the hypoglycemic sulphonylurea gliclazide[34]; the data obtained supported the view that TPA enhanced Ca^{2+}-mobilization from intracellular stores. However, the kinetics of the secretory response to TPA alone have been found to be a slowly rising rate of insulin release[34, 61]; ionophore A23187, which alone gave a rapid but not sustained increase in insulin release, in the presence of TPA gave a secretory response similar to that of glucose[61]. Agents increasing

Table 4. Effects of agents on Ca-phospholipid-dependent protein kinase and on insulin secretion

Agent	Concentration	Ca-PL-PrK-activity (% of control)	Insulin release (% of control)
TPA	0.2 μM	215 ± 6* (5)	358 ± 51* (5)
Vitamin A	100 μM	69 ± 13** (3)	59 ± 7** (3)
Trifluoperazine	40 μM	50 ± 4** (7)	56 ± 6** (44)

The effect of TPA, vitamin A and trifluoperazine on B-cell Ca-PL-PrK activity was assessed under the following conditions: line 1: 1 μM Ca^{2+}, 16 μg/ml phosphatidylserine in the presence or absence of 0.2 μM TPA; line 2: 50 μM Ca^{2+}, 16 μg/ml phosphatidylserine and 1.2 μg/ml diolein in the presence or absence of 100 μM vitamin A; line 3: 50 μM Ca^{2+}, 16 μg/ml phosphatidylserine and 1.2 μg/ml diolein in the presence or absence of 40 μM trifluoperazine. For insulin release batches of rat islets were incubated for 2 h with 10 mM glucose as described in ref. 56 in the presence or absence of 0.2 μM TPA, 100 μM vitamin A or 40 μM trifluoperazine. DMSO was used as a solvent for vitamin A and TPA and was included in control incubations at the same concentration (0.1%). * Significantly greater than control; ** significantly less than control ($p < 0.01$).

intracellular cyclic AMP concentration potentiated the secretory response to TPA plus A23187[61]. These data led to the proposal that Ca-PL-PrK is involved in the second phase of insulin release and allows sustained secretion to occur at lower intracellular Ca^{2+} concentrations[61]. These findings need to be extended to identify specific substrates for Ca-PL-PrK and to show changes in their phosphorylation upon stimulation. Figure 7 shows that incubation of islets with TPA indeed enhanced phosphorylation of protein bands of M_r 17, 22, 35 and 40 kDa (J.M. Lord, unpublished observations). Comparison with figure 6 shows that these bands are of similar M_r to four of the substrates for Ca-PL-PrK detected in islet homogenates.

6. The effect of glucose on protein phosphorylation in intact islets of Langerhans

The interaction of glucose with the pancreatic B-cell leads to changes in Ca^{2+}-fluxes, cyclic AMP concentration and inositol phospholipid metabolism. Hence all three major species of protein kinase described here are potential mediators of the effect of glucose on insulin secretion. As discussed by Christie and Ashcroft[9], effects of glucose on uptake of P_i and on ATP turnover pose problems in the design and interpretation of experiments to assess glucose-induced changes in islet protein phosphorylation; only two such studies have been reported so far. Suzuki et al.[57] claimed to have demonstrated a glucose-induced increase in phosphorylation of polypeptides of M_r 15, 35, 49, 64, 93 and 138 kDa in rat islets. However these effects were small (maximum increase was 22%) and with the protocol used the possibility is not excluded that the changes were a reflection of a glucose-induced increase in specific radioactivity of islet [γ-^{32}P]-ATP. Colca et al.[10] reported that the extent of phosphorylation of the 54-kDa polypeptide they suggest to be β-tubulin was enhanced by glucose. Again however the change was small and no data were given on the specific radioactivity of internal ATP. The authors used a protocol in which medium $^{32}P_i$ was removed prior to stimulation to avoid possible effects of glucose on P_i-uptake. However this procedure does not rule out the possibility of glucose-induced change in [γ-^{32}P]ATP specific radioactivity since glucose is known to increase islet ATP turnover and to induce a loss of internal P_i (the 'phosphate flush'). We do not agree with the authors' contention that stimulation of ATP turnover by glucose would lead to a decrease in ATP specific radioactivity and hence impart a negative bias to their results. On the contrary, if internal $^{32}P_i$ is not at equilibrium with [γ-^{32}P]ATP then enhanced ATP turnover would decrease ATP specific radioactivity only if internal $^{32}P_i$ had a lower specific radioactivity than the [γ-^{32}P] of ATP; this seems highly unlikely. What is needed to resolve this point is direct measurement of the specific radioactivity of the [γ-^{32}P] of islet ATP before and during stimulation by glucose.

An added complication arises from the fact that glucose increases other insular processes such as protein biosynthesis which may involve protein phosphorylation. In our view, the key question of the involvement of protein phosphorylation in glucose-induced insulin secretion presents technical problems which have not so far been satisfactorily overcome.

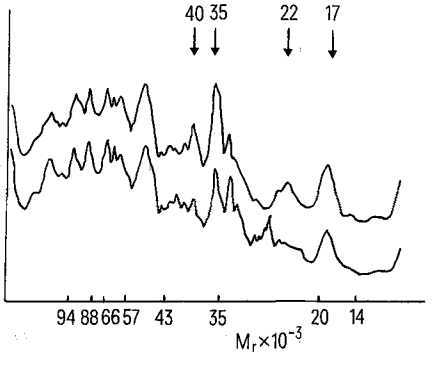

Figure 7. Effect of a tumor promoting phorbol ester (TPA) on islet protein phosphorylation. The effect of TPA on islet protein phosphorylation was determined using the procedure described in the legend to figure 1 (b). The figure shows densitometric scans of the autoradiographs of the 190,000 × g supernatant from islets incubated in the absence (lower trace) or presence (upper trace) of 2 µM TPA. The arrows mark the position of the major bands whose phosphorylation was enhanced by TPA and their M_r values ($\times 10^{-3}$) are given above the arrows.

7. Conclusions

Although this review has concerned itself exclusively with the pancreatic B-cell the hypothesis that protein phosphorylation may be involved in regulation of secretion is of course being widely investigated in many other systems. These include exocrine pancreas[16,43], parotid[16,26,42], platelets[25,27,52], mast cells[53], adrenal medulla[1,3] and nerve tissue[13,37]. Since the molecular basis for exocytosis is far from understood it is perhaps not surprising that, as yet, a coherent pattern has not emerged.

For the B-cell it is striking that nutrient secretagogues such as glucose or experimental procedures such as elevation of extracellular K^+, both of which appear to lead primarily to an increase in intracellular Ca^{2+} activity, are able to initiate insulin release; on the other hand agents which elevate cyclic AMP[9] or agents such as acetylcholine[17] which may primarily activate inositol phospholipid turnover[4] produce little or no stimulation of insulin release unless glucose or other initiator is also present. We are inclined, therefore, to place activation by Ca^{2+} of a Ca^{2+}-calmodulin-dependent protein kinase as central to initiation of release and to ascribe a potentiatory role to activation of cAMP-Prk and Ca-PL-PrK. Although the Ca-PL-PrK is a possible target for Ca^{2+}, current evidence from platelets suggests that diacylglycerol is the physiological regulator of this enzyme[38,39]. If this is the case in the B-cell, then since not all stimulators of insulin release evoke a PI response[4], an alternative Ca^{2+}-sensitive system must be also operative.

Possible targets for Ca^{2+}-calmodulin-dependent protein kinase on the basis of available evidence are myosin, via MLCK, which would form the basis for force-generation for granule movement, and a cytoskeletal component which might be suggested to modulate granular-cytoskeletal interactions and hence translocation. In platelets, it has been found that phosphorylation of

myosin light chains, which occurs on stimulation[27], modifies the ability of myosin to interact with the cytoskeleton[15]. CAMP-PrK and Ca-PL-Prk could exert their proposed amplifying effect via several mechanisms – modification of the sensitivity to phosphorylation of the putative substrate for Ca^{2+}-calmodulin-dependent protein kinase is one possibility. Alternatively or additionally the action of the kinases may be at the level of Ca^{2+}-fluxes to augment the effect of glucose and other initiators. There is growing evidence, reviewed in ref. 30, that ion channels are subject to regulation by protein phosphorylation and modulation of B-cell electrical activity in response to elevation of intracellular cyclic AMP by forskolin has been observed[22].

Based on the above considerations, we propose the scheme in figure 8 as a working hypothesis for the participation of protein phosphorylation in insulin secretion. It is clear that future progress will depend on definitive correlation of changes in insulin release with changes in phosphorylation state of B-cell proteins that can be isolated and identified.

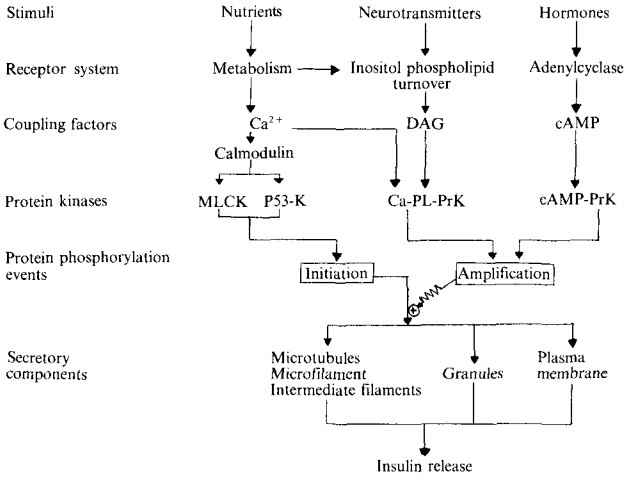

Figure 8. A model for the involvement of protein phosphorylation in the regulation of insulin secretion. The three major classes of insulin secretagogues are suggested to lead to changes in the B-cell concentrations of Ca^{2+}, diacylclycerol (DAG) and cyclic AMP via activation of metabolism, inositol phospholipid breakdown and adenyl cyclase. Ca^{2+}, via calmodulin, activates myosin light chain kinase (MLCK) and the kinase (P53-K) that phosphorylates the endogenous M_r-53,000 protein. The ensuing protein phosphorylation events are regarded as capable of initiating insulin secretion by modification of components of the secretory system which may include cytoskeletal, granule and plasma membrane proteins. Modulation of this pathway to give enhanced or sustained secretion is suggested to be exerted by other phosphorylations catalyzed by Ca^{2+}-phospholipid-dependent protein kinase (Ca-PL-PrK) and cyclic AMP-dependent protein kinase (cAMP-PrK).

Note added in proof. Since completion of this review the following relevant observations have been reported. Vimentin has been shown not to be present in hamster insulinoma cells and the M_r-60,000 phosphopeptide in these cells that undergoes Ca^{2+} influx-induced phosphorylation has been identified as an intermediate filament of the keratin class; its presence in normal hamster islets has been demonstrated using a specific antibody (Schubart, U.K., and Fields, K.L., J. Cell Biol. *98* (1984) 1001–1009). In rat insulinoma Ca^{2+}-phospholipid-dependent protein kinase has been implicated in the phosphorylation of the M_r-29,000 insulin-granule membrane protein (Brocklehurst, K.W., and Hutton, J.C., Biochem. J. *220* (1984) 283–290). Protein kinases in mouse islets have been investigated (Thams, P., Capito, K., and Hedeskov, C.J., Biochem. J. *221* (1984) 247–253): an M_r-53,000 substrate for Ca^{2+}-calmodulin-dependent protein kinase was found in a 27,000 × g-particulate fraction: Ca^{2+}-phospholipid-dependent- and cyclic AMP-dependent protein phosphorylation were observed exclusively in the 27,000 × g-supernatant fraction.

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