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Stimulus–Secretion Coupling in Pancreatic β Cells

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Abstract Insulin secretion is triggered by a rise in the intracellular Ca^{2+} concentration that results from the activation of voltage-gated Ca^{2+} channels in the β-cell plasma membrane. Multiple types of β-cell Ca^{2+} channel have been identified in both electrophysiological and molecular biological studies, but it appears that the L-type Ca^{2+} channel plays a dominant role in regulating Ca^{2+} influx. Activity of this channel is potentiated by protein kinases A and C and is inhibited by GTP-binding proteins, which may mediate the effects of potentiators and inhibitors of insulin secretion on Ca^{2+} influx, respectively. The mechanism by which elevation of intracellular Ca^{2+} leads to the release of insulin granules is not fully understood but appears to involve activation of Ca^{2+}/calmodulin-dependent protein kinase. Phosphorylation by either protein kinase A or C, probably at different substrates, potentiates insulin secretion by acting at some late stage in the secretory process. There is also evidence that small GTP-binding proteins are involved in regulating exocytosis in β cells. The identification and characterisation of the proteins involved in exocytosis in β cells and clarification of the mechanism(s) of action of Ca^{2+} is clearly an important goal for the future.

Key words: pancreatic β cell, insulin secretion, Ca^{2+} channel, exocytosis

INTRODUCTION

Numerous studies have shown that changes in the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, play a central role in the regulation of insulin secretion from pancreatic β cells [see review by Hellman et al., 1992]. This chapter focuses on recent advances in our understanding of the mechanisms that lead to a rise in $[Ca^{2+}]_i$. We also consider the role of Ca^{2+} in the exocytosis of the insulin secretory granules.

The consensus model for stimulus–secretion coupling in the pancreatic β cell may be summarised as follows [Ashcroft and Ashcroft, 1992]. Elevation of the plasma glucose concentration leads to its increased uptake and metabolism within the β cell. Some product of this metabolism, now generally accepted to be ATP, brings about the closure of ATP-sensitive K^+ channels located in the plasma membrane and thereby produces membrane depolarisation. This leads to activation of voltage-dependent Ca^{2+} channels and β-cell electrical activity. The ensuing Ca^{2+} influx elevates the intracellular free Ca^{2+} concentration and initiates exocytosis of insulin-containing secretory vesicles by a mechanism that is not fully understood. Hormones and neurotransmitters may potentiate or inhibit insulin secretion by modulation of stimulus–secretion coupling via effects on ion channels, cytosolic second messengers or the secretory machinery itself.

Glucose and other nutrient secretagogues stimulate insulin secretion as a consequence of their metabolism by the β cell. Detailed accounts of β-cell metabolism can be found elsewhere [Meglasson and Matschinsky, 1986; Ashcroft and Ashcroft, 1992]. Briefly, glucose uptake into the β cell occurs via the facilitative glucose transporter GLUT-2 [Thorens et al., 1988]. The K_m for glucose of this transporter is 50 mM and glucose transport is not rate limiting. The main rate-limiting step for glucose metabolism in β cells is instead phosphorylation of the sugar [German, 1993; Randle, 1992], which is catalysed by the enzyme glucokinase (K_m 10 mM). Glycogen stores are low within β cells under normal conditions. For these reasons the β-cell metabolism acts as an efficient sensor of the extracellular glucose concentration.

Microelectrode recordings of membrane potential in intact islets of Langerhans first established that electrical activity plays a central role in stimulus–secretion coupling in β cells [see

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review by Henquin and Meissner, 1984]. The different ion channels responsible for this electrical activity were subsequently identified and characterised using the patch-clamp technique [Ashcroft and Rorsman, 1990; Ashcroft and Ashcroft, 1992]. These studies have shown that in the absence of glucose, the resting potential of the β cell (~ -70 mV) is principally determined by the activity of ATP-sensitive K^+ channels (K–ATP channels). Closure of these channels by nutrient metabolism leads to a slow depolarisation, which for glucose concentrations that elicit insulin release (> 7 mM) is sufficient to initiate electrical activity. In β cells this has a very distinctive pattern and consists of slow oscillations in membrane potential (known as slow waves) between a depolarised plateau potential on which action potentials are superimposed and a more hyperpolarised interburst interval. Although it is well established that the action potentials result from the activation of voltage-dependent Ca^{2+} channels, the origin of the slow waves is still not completely clear and remains controversial [Ämmälä et al., 1991; Cook et al., 1991]. The mechanism by which glucose modulates the frequency and duration of the slow waves (and thereby the action potentials) is also uncertain.

CALCIUM CURRENTS

Properties

It is well established that a rise in intracellular calcium resulting from calcium influx across the β-cell membrane is the trigger for glucose-induced insulin secretion. As Ca^{2+} entry is primarily controlled by voltage-gated Ca^{2+} channels, the identification and characterisation of these channels is of importance for understanding β-cell stimulus–secretion coupling. There is growing evidence that the β cell possesses a heterogeneity of Ca^{2+} channels and that the expression of the different channel types may vary between species. Modulation of β-cell Ca^{2+} channels by a number of secretagogues and cytosolic second messengers has been described, but again appears to differ between different species and between cell lines and primary cultured β cells.

To date, two types of voltage-dependent Ca^{2+} channel have been described in single-channel studies [see Ashcroft and Rorsman, 1989, for review], with properties resembling those of L-type and T-type channels in other tissues [Nowcky et al., 1985]. The T-type (low voltage-activated or slow-deactivating) channel is activated at potentials around -50 mV, exhibits voltage-dependent inactivation and has a single-channel conductance of 8 pS with 110 mM external Ba^{2+} as the charge carrier [Ashcroft et al., 1990]. The L-type channel (high-voltage activated or fast-deactivating channel) is distinguished by its sensitivity to the dihydropyridines (DHP), being inhibited by DHP antagonists such as nifedipine and potentiated by DHP agonists like BAYK 8644. It is activated at potentials around -40 mV, inactivation of the current is Ca^{2+} dependent, and the single-channel conductance is 20–25 pS with 110 mM external Ba^{2+} [Smith et al., 1993]. Mouse β cells appear to possess only the L-type Ca^{2+} channel [Rorsman et al., 1988; Smith et al., 1993], whereas T-type channels have been identified at the single-channel level in rat [Ashcroft et al., 1990; Sala and Matteson, 1990), human [Smith and Quayle, 1993] and RINm5F [Velasco, 1987] β cells.

In general, single Ca^{2+} channel recordings are carried out using a high extracellular Ba^{2+} concentration to increase the single-channel current amplitude. The high concentration of divalent cations screens negative surface charges on the membrane and so shifts the voltage dependence of the channel to more positive potentials. The extent of this shift has been quantified for the L-type Ca^{2+} channel by Smith et al. [1993] and is around 30 mV when using 100 mM Ba^{2+} instead of pseudo-physiological solutions (2.6 mM Ca^{2+}). These studies also demonstrated that single L-type Ca channel openings occur at the β-cell resting potential in low extracellular divalent cation solutions. This suggests that Ca^{2+} influx through these channels may contribute to the background Ca^{2+} influx into the β cell and would explain why DHPs can modulate basal insulin secretion [Al-Mahood et al., 1986; Boschero et al., 1990].

New evidence shows that multiple types of high voltage-activated (HVA) Ca^{2+} channels may exist in β cells. First, dihydropyridines do not completely inhibit the HVA current in RINm5F cells, indicating that DHP-*in*sensitive HVA Ca^{2+} channels are present, in addition to the DHP-sensitive L-type Ca^{2+} channels [Pollo et al., 1993]. The DHP-insensitive current activates at a slightly more negative membrane *potential*, but the similarity in voltage-dependence means that it is difficult to separate from the L-type current except by pharmacological means. The

toxin ω-conotoxin, which has been used to identify N-type Ca^{2+} channels, only partially blocks the DHP-insensitive current, indicating that this current is carried by both ω-conotoxin sensitive Ca^{2+} channels and Ca^{2+} channels which are insensitive to both ω-conotoxin and dihydropyridines. High-affinity binding sites for ω-conotoxin have also been identified in RINm5F cells [Sher et al., 1992]. The DHP-insensitive Ca^{2+} channels appear to constitute about 45% of the total HVA Ca^{2+} current in RINm5F cells but are present at a much lower relative density (20%) in human β cells [Pollo et al., 1993]. Differential expression of heterogeneous HVA Ca^{2+} currents may explain some of variation reported for the effects of channel modulators in different types of β cell (see below).

Evidence for multiple kinds of HVA Ca^{2+} channels also comes from molecular biological studies. The L-type Ca^{+} channel of skeletal muscle is composed of 5 distinct subunits (α_1, α_2, β, γ, δ). Molecular cloning has shown that the α_1-subunit functions both as the Ca^{2+} channel pore and as the DHP receptor [Campbell et al., 1988]. This subunit comprises 4 repeated domains each of which contains 6 putative transmembrane segments (S1–S6) and a pore-forming region known as H5. Two isoforms of the α_1-subunit of the L-type Ca^{2+} channel have been identified in β cells [Perez-Reyes et al., 1990; Seino et al., 1992]. One of these is 98% homologous to the cardiac-type α_1-subunit, and the other isoform shares 98% identify with the neuroendocrine-type α_1-subunit. A number of splice variants of the latter have been reported to be expressed in HIT cells [Perez-Reyes et al., 1990].

Modulation by Kinases

It is well established that L-type Ca^{2+} channels in a variety of tissues are potentiated by activation of cyclic AMP-dependent protein kinase (PKA). In β cells, agents that elevate cyclic AMP (cAMP) reduce the rate of Ca^{2+} channel inactivation without substantially altering the peak amplitude of the Ca^{2+} current [Ämmälä et al., 1993a]. These effects may contribute to the increased spike amplitude and longer slow waves found in the presence of agents that increase cAMP [Henquin, 1985; Henquin et al., 1987]. Ca^{2+} entry (as assessed by the integrated Ca^{2+} current) is increased by about 70% following elevation of cAMP. Since the effect of cAMP is reversed by the specific PKA inhibitor of Rp-cAMPs, it appears that PKA activation mediates the effect of the nucleotide [Ämmälä et al., 1993a]. It seems likely that glucagon and other secretagogues, which elevate cAMP in β-cells, will also be found to increase the β-cell Ca^{2+} current.

The Ca^{2+}-calmodulin-dependent protein kinase II (CAM kinase II) inhibitor KN-62 blocks the L-type Ca^{2+} channel in β cells and HIT cells [Li et al., 1993; Ämmälä et al., 1993]. However, two lines of evidence suggest that this effect is not mediated by inhibition of CAM kinase II. First, KN-62 blocked Ca^{2+} influx under conditions in which CAM kinase II is expected to be fully activated and thus insensitive to the inhibitor [Li et al., 1993]. Second, the calmodulin-binding domain of CAM-kinase II (residues 290–309), a potent and specific inhibitor of the enzyme, was without effect on L-type Ca^{2+} currents [Ämmälä et al., 1993b]. This suggests that KN-62 may act as a direct blocker of the β-cell Ca^{2+} channel and that CAM kinase II does not influence β-cell Ca^{2+} currents.

Protein kinase C (PKC) also modulates Ca^{2+} channels in β cells. In perforated patch recordings from mouse β cells, the phorbol ester PMA, which directly activates PKC, increases the peak amplitude of the Ca^{2+} current by about 30% but does not influence inactivation of the current [Ämmälä et al., submitted]. A number of membrane permeable analogues of diacylglycerol, which activate PKC, have also been reported to influence whole-cell and single-channel Ca^{2+} currents. For example, DiC10 increases the activity of single L-type Ca^{2+} channels in RINm5F cells [Velasco and Petersen 1989], whereas DiC8 inhibits whole-cell Ca^{2+} currents in mouse β cells [Plant, 1989], and both DiC10 and diacylglycerol have slight inhibitory effects on single L-type Ca^{2+} channel currents [Smith et al., 1991]. It seems possible that the synthetic diacylglycerols mediate their effects via a mechanism that is not associated with activation of PKC [see also Hockberger et al., 1989].

Modulation by GTP-Binding Proteins

A slowly developing (1–2 min) inhibition of the whole-cell Ca^{2+} current in mouse β cells was observed following intracellular application of the nonhydrolysable GTP analogue, GTPγS, by liberation from a caged precursor [Ämmälä et al., 1992]. This effect persisted in the presence of cAMP, indicating that it did not result from inhibition of adenylate cyclase, and was not mimicked by GTP, or prevented by inclusion of

GDPβS in the pipette solution or pretreatment with pertussis toxin. Indeed, pertussis toxin caused an increase in the Ca^{2+} current, suggesting that the current is subject to partial resting inhibition by G proteins.

Many inhibitors of insulin secretion are known to mediate their actions by interacting with GTP-binding proteins. In HIT cells, epinephrine reversibly blocks whole-cell Ca^{2+} currents [Keahey et al., 1989], and similar results are found with somatostatin [Hsu et al., 1989] and galanin [Homaidan et al., 1991]. Inhibition by the first two agents appears to involve a G protein, since it is abolished by preincubation of the cells with pertussis toxin. In addition, adrenergic inhibition is irreversible in the presence of intracellular GTPγS and is abolished by the inclusion of GDPβS in the intracellular solution [Keahey et al., 1989]. By contrast, catecholamines and galanin appear to be without effect on L-type Ca^{2+} channels in mouse β cells [Bokvist et al., 1991], suggesting there may be species differences. Indeed, in mouse β cells the inhibitory action of epinephrine, galanin, and somatostatin on electrical activity is due to activation of a low-conductance G protein coupled K^+ channel [Rorsman et al., 1991].

Modulation by Diabetic Serum

Serum isolated from newly diagnosed insulin-dependent (IDDM) diabetics produces a marked up-regulation of L-type Ca^{2+} channel activity in mouse β cells and RINm5F cells [Juntti-Berggren et al., 1993]. The effect required overnight culture in the presence of serum, indicating that the antibody does not act as a direct activator of the channel. Of considerable interest was the finding that incubation with IDDM serum produced β-cell death, which could be prevented by incubation with L-type Ca^{2+} channel blockers, suggesting that it resulted from an increased Ca^{2+} influx. This finding may be of relevance to β-cell destruction in IDDM: thus, lymphocyte infiltration may be the consequence, rather than the cause, of β-cell destruction [Christie, 1992].

EXOCYTOSIS

Elevation of intracellular Ca^{2+} leads to the release of insulin by a mechanism that is still poorly understood. This process, known as exocytosis, resembles that of other neuroendocrine cells [Burgoyne et al., 1991]. This section reviews current knowledge of exocytosis in β cells.

Methods

Two main methods have been used to study exocytosis from single β cells. In the first of these high-voltage discharge, detergents or certain toxins are employed to permeabilise populations of β cells and so enable the cytosolic environment to be controlled. Secretion is then measured by assay of released insulin. The second technique uses the fact that the cell capacitance is proportional to the cell surface area and increases when secretory vesicles fuse with the plasma membrane. A variant of the patch-clamp method, which measures changes in cell capacitance, may thus be used to monitor exo- and endocytosis from *single* cells [Neher and Marty, 1982]. This method has greater temporal resolution (10 measurements/sec) and can be used to monitor exocytosis in real time. Thirdly, release of granule contents may be followed in real time by monitoring the loss of a fluorescent dye, such as quinacrine, previously loaded into the secretory vesicle [Pralong et al., 1990]. Since the loss of quinacrine fluorescence is correlated with changes in cell capacitance, it is reasonable to conclude that the capacitance method does indeed monitor release of the insulin secretory granule.

Ca Dependence of Release

Glucose-stimulated insulin secretion is dependent on extracellular Ca^{2+} influx [see Hellman et al., for review]. Patch-clamp recordings from single β cells have shown that the voltage dependence of the Ca^{2+} current, $[Ca^{2+}]_i$ transient and exocytosis all possess the same bell-shaped voltage-dependence, being initiated around −20 mV, maximal around +10 mV and declining to zero at potentials positive to +40 mV [Gillis and Misler, 1992; Ämmälä et al., 1993b].

It is clear that when the Ca^{2+} channel opens there will be a rapid rise in $[Ca^{2+}]_i$ localised to the vicinity of the Ca^{2+} channel, resulting in a steep spatial gradient of Ca^{2+}. The secretory granules that are ready to be released will be located close to the plasma membrane and will therefore be exposed to this high Ca^{2+} concentration. The difficulty in determining the Ca^{2+} dependence of exocytosis, using fluorescent Ca^{2+} indicators, is that only a fraction of the dye is present in these zones of high Ca^{2+}. Most of the indicator does not sense the submembrane Ca^{2+} concentration because it lies within the cell. This means that the average cytosolic Ca^{2+} re-

ported by the dye is considerably lower than that at the exocytotic release sites. This explains why, for the same measured average $[Ca^{2+}]_i$, the rate of exocytosis in response to voltage-gated Ca^{2+} entry is much higher than that elicited by perfusion of the cell with Ca^{2+} buffer [Ämmälä et al., 1993], as has previously been shown for chromaffin cells [Augustine and Neher, 1992].

The unitary events that result from the fusion of single secretory granules with the plasma membrane have also been recorded [Ämmälä et al., 1993]. Both exo- and endocytoses had a mean amplitude of about 2 fF, corresponding to a mean vesicle diameter of about 250 nm. This compares well with the size of the secretory granule measured by electron microscopy, which has a diameter of 200–300 nm [Dean, 1973; Hutton, 1989].

A strong depression of the secretory response is seen during a train of depolarising stimuli [Ämmälä et al., 1993b]. A similar phenomenon has also been described in other secretory cells [Thomas et al., 1993] and is usually attributed to the rapid depletion of a readily releasable pools of secretory granules. Replenishment of this pool must occur during the intervals between trains of pulses as exocytosis can be elicited by a second train applied some seconds later. Under some conditions, facilitation of the secretory response may also be observed [Ämmälä et al., 1993b].

Exocytosis may occur as fast as 500 granules/sec, that is, about 5% of the secretory granule population/sec [Ämmälä et al., 1993b]. However, this rate of exocytosis can only be sustained for short periods (50 msec) and declines rapidly during repetitive stimulation. The rate of insulin release in biochemical measurements ($<$ 1–5% of the granule population/min) [Jones et al., 1989; Li et al., 1992; Bergsten and Hellman, 1992] corresponds more closely to the rate of endocytosis measured with the capacitance method ($\sim$ 4%/min) [Ämmälä et al., 1993b]. This suggests that membrane retrieval may set an upper limit on the rate of exocytosis during extended periods of secretion.

During standard whole-cell recordings, the ability of voltage-activated Ca^{2+} influx to elicit exocytosis declines with time and is usually lost within 10 min [Ämmälä et al., 1993b]. A similar phenomenon is found for permeabilised β cells, where only the first of a series of Ca^{2+} pulses is able to elicit insulin release [Jones et al., 1992]. A marked decline in Ca^{2+}-dependent protein phosphorylation (in particular of a Mr 54-kD peptide) has been reported to correlate with this loss of secretory sensitivity [Jones et al., 1992]. Since exocytosis may be preserved for $>$ 1 hr in perforated patch recordings, one possibility is that some diffusible cytosolic component necessary for exocytosis is dialysed from the cell in permeabilised cells and standard whole-cell recordings. However, both cAMP and PMA were able to stimulate release from permeabilised β cells which were no longer Ca^{2+} responsive, suggesting the exocytotic machinery remains intact [Jones et al., 1992].

Role of CAM kinase

Our understanding of how an increase in intracellular Ca^{2+} initiates insulin secretion is very limited. The available data are consistent with the view that calmodulin represents an important mediator of effects of Ca^{2+} on the secretory process via activation of a Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) [Harrison and Ashcroft, 1982; Harrison et al., 1984].

CAM kinase is found in various insulin-secreting tissues including human islets of Langerhans [Harrison et al., 1984]. It is also present in neurones and neuroendocrine cells where it appears to play a role in transmitter release. The β-cell CaM kinase requires calmodulin for activity, is sensitive to low concentration of Ca^{2+} and phosphorylates an endogenous protein with a molecular mass of 53-kD on SDS gels [Harrison and Ashcroft, 1982]. In intact islets, the activity of CaM kinase was correlated with glucose-stimulated insulin secretion [Colca et al., 1983b]. Dehydrouramil (DHU), a stable analogue of alloxan, specifically inhibits β-cell CaM kinase in extracts of islets and, in intact islets, blocked both glucose-stimulated insulin secretion and the potentiatory effects of activating either protein kinase A or C [Harrison et al., 1986; Li et al., 1992]. Similar data were obtained with alloxan [Colca et al., 1983a]. Direct evidence for a central role for CaM kinase in β-cell secretion has also been obtained using the capacitance method to measure exocytosis from single β cells [Ämmälä et al., 1993b]. We found that the calmodulin binding domain of CaM kinase II (residues 290–309, 1 μM), which is a specific inhibitor of the enzyme, was able to substantially reduce exocytosis when included in the pipette solution. There was no effect on the Ca^{2+} current indicating that the kinase acts at a later stage in the secretory pathway, distal to eleva-

tion of cytosolic Ca^{2+}. Taken together, these data suggest a central role for CaM kinase in the initiation of insulin release [Harrison et al., 1986].

The target(s) for CAM kinase is unknown, but one has been proposed to be a subunit of tubulin [Colca et al., 1983a] and therefore involved in the regulation of the interaction between the secretory vesicles and the cytoskeleton. A major substrate for CaM kinase II in nervous tissue is synapsin I, which is bound to a high-affinity site on synaptic vesicles [Huttner et al., 1983] and may link the vesicles to the cytoskeleton [Petrucci and Morrow, 1987]. An increase in cytosolic Ca^{2+} stimulates CAM kinase II dependent phosphorylation of synapsin I and thereby produces mobilisation of synaptic vesicles [Llinás et al., 1991]. It is therefore possible that in β cells CAM kinase fulfills a similar functional role, although it is unlikely to interact with synapsins, as there is no evidence for these proteins in β cells.

Role of cAMP

Agents that increase cytosolic cAMP markedly enhance stimulus–secretion coupling in β cells both via stimulation of Ca^{2+} influx (and consequently $[Ca^{2+}]_i$) and by a direct effect on the secretory machinery itself. Both effects are mediated by activation of protein kinase A, as they can be reversed by Rp-cAMPs, a specific inhibitor of this enzyme [Ämmälä et al., 1993a; Persaud et al., 1990]. Patch-clamp studies have shown that cAMP increases Ca^{2+} influx primarily by slowing inactivation of the L-type Ca^{2+} channel, as described above. The resulting increase in Ca^{2+} influx elevates intracellular Ca^{2+} and thereby potentiates exocytosis. However, these increases in average cytosolic Ca^{2+} are small and it has been estimated that only about 20% of the effects of cAMP on exocytosis result from elevation of $[Ca^{2+}]_i$ [Ämmälä et al., 1993a]. There is good evidence that the primary mechanism by which cAMP enhances insulin secretion is independent of its action on cytosolic Ca^{2+}. First, cAMP stimulates insulin from permeabilised β cells in which the Ca^{2+} concentration is buffered at a constant level [Jones et al., 1986]. Second, electrophysiological methods of measuring secretion from single β cells have demonstrated that cAMP can stimulate exocytosis at levels of Ca^{2+} that do not initiate release and can accelerate the rate of exocytosis at higher $[Ca^{2+}]_i$ [Ämmälä et al., 1993a; Gillis and Misler, 1993]. The depression of exocytosis during a train of depolarising pulses is also reduced by agents that elevate cAMP [Gillis and Misler, 1993]. Another finding of interest is that cAMP is also able to stimulate exocytosis following exhaustion of the response to a train of depolarising voltage pulses [Ämmälä et al., 1993]. One explanation of these findings is that cAMP extends the distance from the Ca^{2+} channels over which the secretory granules can be recruited, by increasing the Ca^{2+} sensitivity of exocytosis. Thus, cAMP might directly affect granule mobilisation.

The role of cGMP in insulin secretion has recently attracted some attention, as it is the one of the products that mediates the action of nitric oxide. However, there was no effect of elevation of cGMP on K-ATP channel activity (Ashcroft, unpublished observations), on intracellular Ca^{2+}, (Ämmälä and Rorsman, unpublished observations) or on exocytosis itself measured using the capacitance method [Ämmälä et al., 1993a].

Protein kinase A has been detected and characterised in a variety of β-cell preparations [for review, see Harrison et al., 1984; Hughes and Ashcroft, 1992]. Type I and II isoenzymes, with properties similar to those in other tissues have been identified in rat islets [Sugden et al., 1979]. Rapid changes in the phosphorylation of several β-cell peptides in both the cytosolic and particulate fractions have been demonstrated in islets stimulated by elevation of cAMP [Hughes and Ashcroft, 1992]. As for CAM kinase II, the key substrate(s) for protein kinase A is unknown. However, several proteins involved in granule mobilisation and exocytosis may be considered, for example: (1) PKA-dependent depolymerisation of the submembrane actin network, such as that proposed for adrenal chromaffin cells [Burgoyne, 1991]; and (2) PKA-dependent phosphorylation of a synapsin-like protein leading to mobilisation of the secretory granules, as occurs for neuronal secretion [Llinás et al., 1991].

It is well established that hormones such as glucagon potentiate insulin secretion by increasing the β-cell cAMP concentration. Studies with single β cells purified by fluorescence-activated cell sorting (FACS) have suggested that cAMP may also have a crucial role in glucose-stimulated insulin secretion from isolated β cells [Van Schravendijk et al., 1990; Pipeleers, 1992; Wang et al., 1993]. Thus, glucose-stimulated insulin release from FACS purified β cells can only be

elicited in the presence of agents which elevate intracellular cAMP or in the presence of glucagon-secreting α cells. Furthermore, exocytosis from intact single β cells measured with the capacitance method is minimal in the absence of cAMP-increasing agents [Ämmälä et al., 1993a]. These studies suggest that elevation of cAMP may be essential for glucose-stimulated insulin secretion from β cells: the lack of a requirement for experimentally induced cAMP elevation in glucose-stimulated insulin secretion from intact islets may be explained by a paracrine action of glucagon released from adjacent islet α cells. However, this hypothesis is hard to equate with the report that glucose is still able to stimulate insulin secretion from islets exposed to a cAMP analogue (Rp-cAMPs) that completely abolishes protein kinase A activity [Persaud et al., 1990].

Role of Protein Kinase C

Protein kinase C (PKC) is activated by diacylglycerol, generated in intact β cells via receptor-linked phospholipase C by a number of potentiators of insulin secretion, including acetylcholine and bombesin [see reviews by Persaud et al., 1992; Morgan and Montague, 1992]. Diacylglycerol is also produced following increases in $[Ca^{2+}]_i$ due to Ca^{2+}-dependent activation of phospholipase C, and thus PKC will activated under all conditions in which intracellular calcium is elevated [Biden et al., 1987]. For example, glucose and other nutrient secretagogues activate PKC in this way.

Protein kinase C has been purified to homogeneity in β cells and exists as a monomer of 80 kD [Lord and Ashcroft, 1984]. A number of endogenous substrates for PKC have been identified in rodent β cells [Harrison et al., 1984; Hughes and Ashcroft, 1988], and there is some evidence that two of these, a 29-kD protein [Dunlop and Larkins, 1986] and a 37-kD protein located in the particulate fraction [Hughes and Ashcroft, 1988], may be involved in insulin secretion.

Protein kinase C activation markedly potentiates insulin secretion [Persaud et al., 1992]. Thus tumour promoting agents such as TPA, which activate protein kinase C, stimulate insulin release [Harrison et al., 1984; Howell et al., 1990]. Furthermore, clomiphene, a potent inhibitor of PKC, blocks the effect of TPA on insulin release and protein phosphorylation in intact islets [Hughes and Ashcroft, 1988]. Likewise, the PKC inhibitor H7 blocks TPA-stimulated insulin secretion [Metz, 1988].

It appears that activation of protein kinase C is not an absolute requirement for glucose-stimulated insulin secretion, since islets in which PKC has been down-regulated essentially completely (by long-term treatment with phorbol esters) still retain a secretory response to glucose [Metz, 1988; Howell et al., 1990; Hughes et al., 1990]. Instead, PKC appears to be involved in the stimulation of insulin secretion by potentiators of release. Thus, there is good evidence that activation of PKC is primarily responsible for the stimulatory effects of acetylcholine, and the muscarinic agonist carbamylcholine (CCh), on insulin release. First, CCh generates diacylglycerol in islets by activation of phospholipase C [Peter-Riesch et al., 1988] and stimulates translocation of PKC from the cytosol to the plasma membrane [Regazzi and Wollheim, 1990; Persaud et al., 1989]. Second, the ability of acetylcholine to potentiate insulin release is significantly reduced, although not completely abolished, in islets in which PKC activity was down-regulated completely [Hughes et al., 1990; Persaud et al., 1991].

The mechanism by which PKC promotes exocytosis remains unresolved. The principal effect of this kinase appears to be to sensitise the secretory machinery to Ca^{2+}, as has been demonstrated in intact HIT cells [Hughes et al., 1990], in permeabilised islets [Jones et al., 1988] and in single mouse β cells by the capacitance method [Ämmälä et al., submitted]. Elevation of $[Ca^{2+}]_i$ by Ca^{2+} influx is probably not important, as the effect of PKC activation on the β-cell Ca^{2+} current is small [Ämmälä et al., 1994]. Likewise, mobilisation of Ca^{2+} from intracellular stores is not important because TPA is able to promote insulin secretion while *lowering* intracellular Ca^{2+} [Arkhammar et al., 1989; Hughes et al., 1992]. In perforated patch recordings, the effects of activation of PKC and activation of PKA on exocytosis are additive [Ämmälä et al., submitted]. Furthermore, whereas the phosphatase inhibitor okadaic acid strongly potentiates forskolin-stimulated release, it has no effect on exocytosis stimulated by PMA. This suggests that although PKA and PKC stimulate exocytosis in a similar fashion, they may do so by phosphorylating different substrates.

In addition to sensitising the secretory machinery to Ca^{2+}, PKC is able to modulate Ca^{2+}-independent insulin release, since the phorbol ester PMA elicited release from permeabilised RIN m5F cells in the absence of $[Ca^{2+}]_i$ [Regazzi

et al., 1989] and in Ca^{2+}-insensitive permeabilised β cells [Jones et al., 1992].

Role of GTP-Binding Proteins

There is accumulating evidence that $[Ca^{2+}]_i$ is not the only factor regulating exocytosis in β cells. In electrically permeabilised RINm5F cells [Valler et al., 1987] and islet cells [Wollheim et al., 1987] Ca^{2+}-independent insulin secretion can be stimulated by the nonhydrolysable GTP analogues GTPγS and Gpp(NH)p. This effect could not be attributed to activation of protein kinases, since no concomitant changes in phosphoprotein pattern were observed [Regazzi et al., 1989]. One possible target for GTPγS is Ge, a putative GTP-binding protein involved in exocytosis [Lindau and Gomperts, 1991]. GTP also appears to mediate inhibition of Ca^{2+}-stimulated insulin secretion at a late stage in stimulus–secretion coupling, following activation of adrenergic, somatostatin or galanin receptors [Ullrich and Wollheim 1988, 1989], which may suggest the involvement of more than one G protein in the exocytotic process.

In other cell types, a variety of monomeric small GTP-binding proteins (SMGs) have been implicated in exocytosis. A synthetic peptide corresponding to the effector domain of one of these proteins, rab3a, has been shown to reconstitute exocytosis in permeabilised chromaffin cells [Senyshyn et al., 1992] and pancreatic accinar cells [Padfield et al., 1992] and to promote secretion in mast cells [Oberhauser et al., 1992]. The presence of SMGs has been demonstrated in the insulin-secreting cell lines RINm5F and HIT T15 [Regazzi et al., 1992b]. About 10 ras-related SMGs were detected. Seven of these were associated with the secretory granule-enriched fraction, and of one these was also detected by a monoclonal antibody to rab3a. rab3a thus emerges as a new candidate for the regulation of exocytosis in β cells. Indeed a synthetic peptide to rab3a stimulates insulin secretion from permeabilised HIT cells (Li G, Balch WE, and Wollheim CB, unpublished observations). Rho-related SMG proteins were also detected in HIT cells [Regazzi et al., 1992b].

According to the model proposed by Bourne [1988], SMGs cycle between a soluble and a membrane-attached state. The binding of both ras and rho-related proteins to membranes appears to be controlled by interaction with a regulatory protein known as a GDP dissociation inhibitor. There is evidence that most SMGs in the cytosol of insulin-secreting cells are complexed to GDP dissociation inhibitor proteins in a 1:1 fashion [Regazzi et al., 1992a].

Role of Cytoskeleton

A well-developed cytoskeleton consisting of microtubules and microfilaments exists in β cells and is believed to play a role in the movement of secretory granules to the plasma membrane [see review by Howell, 1984]. Evidence that microtubules play a role in insulin secretion comes from two observations. First colchicine retards the movement of insulin from its site of synthesis to the plasma membrane [Pipeleers et al., 1976; Malaisse-Lagae et al., 1979]. Second, glucose-stimulated insulin secretion is decreased, but not abolished, in β cells preincubated for 2 hr with 1 μM colchicine [Boyd, 1982]. Although colchicine has multiple effects, this dose is three orders of magnitude lower than that which alters glucose uptake and ATP activity or disrupts the Golgi apparatus. These data suggest that microtubules may be involved in replenishment of a readily releasable granule store. An extensive submembrane network of actin filaments, 50–300 nm thick, is also present in β cells [Orci et al., 1972; see review by Boyd, 1988]. Cytochalasin B prevents F-actin polymerisation, and at 10-μM concentration potentiates insulin secretion. This observation, and the absence of secretory granules in the submembrane actin network, have led to the suggestion that the actin network acts as a barrier to exocytosis [Orci et al., 1972].

Endocytosis

Following exocytosis, the secreted membrane must be retrieved or the cell will swell. Little is known about the retrieval of membrane secreted via the regulated pathway in any cell type and the mechanisms controlling endocytosis in β cells are completely unknown.

In capacitance recordings from β cells using the standard whole-cell configuration, endocytosis is rarely observed to follow exocytosis elicited by depolarisation [Ämmälä et al., 1993b]. By contrast, membrane retrieval is seen much more frequently in perforated patch recordings [Ämmälä et al., 1993b]. One explanation for these findings is that some cytosolic component necessary for preservation of endocytosis is dialysed from the cell in the standard whole-cell configuration. Alternatively, it is possible that some component of the pipette solution inhibits

endocytosis in standard whole-cell recordings. This substance(s) is likely to be of low molecular weight as endocytosis is absent, even at the start of standard whole-cell recordings, indicating that diffusion occurs rapidly.

In chromaffin cells, there is evidence that the requirements for endocytosis are quite different from those regulating exocytosis [von Grafenstein and Knight, 1993]. In particular, it appears that endocytosis can proceed in the virtual absence of Ca^{2+} and Mg ATP. Whether this is also the case for membrane retrieval in β-cells remains to be established.

CONCLUDING REMARKS

In this chapter, we have tried to review recent work on the mechanisms underlying the role of calcium in stimulus–secretion coupling in β cells. It is clear that considerable progress has been made in our understanding of the properties of the β-cell Ca^{2+} currents, and the cloning of one of these channel proteins now offers the opportunity for studies on the relationship between channel structure and function. The basic properties of exocytosis have been clarified but still nothing is known about this process at the molecular level. Clearly, an important area of research in the future will be the identification and characterisation of the proteins involved in exocytosis and further clarification of the mechanism(s) of action of Ca^{2+}.

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