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Review Articles

Calmodulin and Insulin Secretion

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Insulin secretion is directly stimulated by a wide variety of agents, both endogenous and exogenous [1-4]. Glucose is probably the major insulin secretagogue but amino acids, glucagon and gastrointestinal hormones all stimulate insulin release from the B cells of the pancreatic islets; drugs such as the sulphonylureas and changes in extracellular potassium concentrations also influence the release of insulin. The mechanisms by which these widely differing substances stimulate insulin secretion are not totally clear. However, there is evidence that both adenosine 3′,5′ cyclic monophosphate (cyclic AMP) and calcium ions function as signal molecules linking the recognition of the stimulus to the secretory process.

There is considerable evidence to suggest that the effects of hormones, such as glucagon and secretin, are mediated through an increase in the intracellular cyclic AMP concentration. However, these agents and other substances such as prostaglandins, phosphodiesterase inhibitors and cholera toxin (which also increase cyclic AMP) do not influence insulin secretion in the absence of a primary stimulus such as glucose. While glucose has been found to increase cyclic AMP concentrations in some studies, there appears to be no correlation between insulin secretion and cyclic AMP levels or between the time courses of the two events. It has been suggested that the effect of glucose on cyclic AMP may be secondary to its effect on calcium fluxes or mobilisation.

Calcium and Insulin Secretion

The release of insulin requires the presence of extracellular calcium ions [5, 6]. In response to glucose, depolarization of the B cell membrane is accompanied by Ca^{2+}-dependent action potentials [7]. The resulting increase in the intracellular accumulation of calcium ions appears to trigger the release mechanism [8]. The secondary stimuli do not appear to alter calcium uptake but may act by affecting the intracellular distribution of the cation by causing the release of calcium ions from an intracellular pool [9]. This effect may be secondary to the increased accumulation of cyclic AMP. It seems clear, however, that any redistribution of calcium ions is insufficient to trigger release, since these secondary stimuli (or modifiers) only stimulate release in the presence of glucose or other metabolites [10, 11].

Calmodulin [12-14]

Thus, the intracellular concentration of calcium ions appears to play a major role in the insulin secretory process. How calcium ions might effect their regulatory functions has become clearer in the last 2-3 years with the recognition that calmodulin, the calcium-dependent regulatory protein, has a pivotal role in mediating calcium-dependent cellular processes.

Calmodulin was first discovered over 10 years ago as an activator of brain cyclic nucleotide phosphodiesterase. Subsequently, several investigators described similar activators in different tissues from many species. It eventually became clear that these various activators were in fact the same protein which has come to be known as calmodulin. This protein is now recognised as a member of the so-called 'troponin-C-superfamily' of calcium binding proteins which have a fundamental role in muscle contraction and relaxation and probably in intestinal calcium transport.

Calmodulin has a number of interesting physical properties (Table 1) not least being its remarkable resistance to denaturation by boiling, by urea, and by detergents such as sodium dodecyl sulphate. Indeed it will bind calcium even in the presence of 1% sodium dodecyl sulphate or 6 mol/l urea. Its structure has been remarkably conserved not only in the animal

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Table 1. Properties of calmodulin

- Straight chain polypeptide
- Molecular weight: 16,500–16,700
- Isoelectric point, pH approximately 4
- Calcium binding, four sites; affinity, approximately 10^{-6} molar
- Binds to phenothiazines – calcium dependent
- Rich in phenylalanine
- No cystein or tryptophan content
- Lysine at position 115 is trimethylated

Table 2. Some cellular regulatory activities of calmodulin

- Cyclic nucleotide metabolism
- Calcium transport
- Glycogen metabolism
- Microfilament/microtubule functions
- Myosin light chain kinase
- Protein phosphorylation

kingdom from earthworm and octopus to pig and ox but also in the plant kingdom where spinach and peanut calmodulin differ little from each other and from that found in the animal species; only seven conservative amino acid substitutions (out of 148 amino acids) have been noted between the sea pansy, rat and ox. Moreover recent studies using recombinant DNA techniques are consistent with conservation of the base triplets that code for the amino acids, amongst species as diverse as electric eel, chick, man and wheat. This remarkable resistance to change in a molecule over millions of years in evolutionary time is shared only with the histone proteins (a major constituent of nuclear chromatin) and would seem to argue for a crucial role in cellular metabolism.

That calmodulin is important in several metabolic processes (Table 2) is now well established. It influences glycogen metabolism, the microfilament and microtubules system (important in intracellular transport and cell division) and is linked with the cyclic nucleotide second messenger system through its effects on cyclic nucleotide synthesis (adenylate cyclase) and degradation (phosphodiesterase), and its effects on protein phosphorylation.

A number of neuroleptic drugs have been found to reversibly bind calmodulin in a calcium-dependent manner and to inhibit its action [15]. Among the most potent of these drugs are the phenothiazine tranquillizers. Trifluoperazine, notably, shows Ca^{2+}-dependent reversible binding to two high affinity binding sites on calmodulin. This binding is accompanied by potent inhibition of the Ca^{2+}-calmodulin dependent activation of cyclic nucleotide phosphodiesterase. Other calcium binding proteins like troponin-C and S100 protein from brain also bind to phenothiazines but to a lesser extent than calmodulin. This relative specificity of phenothiazines to bind and inhibit calmodulin-dependent phosphodiesterase has been used to define a possible role for calmodulin in stimulus-secretion coupling particularly in insulin secretion.

Calmodulin in the B Cell

It has been shown that extracts of rat pancreatic islets prepared by sonication contain a heat-stable component which activates bovine brain phosphodiesterase [16]. Maximal activation achieved by this factor was sixfold relative to basal activity which was equivalent to that observed with authentic calmodulin. Moreover, like calmodulin, the activation by the islet cell factor was inhibited by trifluoperazine and was abolished in the absence of calcium. A protein sharing molecular and biological characteristics with calmodulin has been isolated from canine pancreas [17]. More recently sufficient tissue from a rat islet cell tumour has been obtained to extract and purify a protein having the same electrophoretic mobility, calcium-binding characteristics and amino acid composition as rat and bovine brain calmodulin [18]. It seems clear therefore that calmodulin is present in insulin secretory cells.

A Role for Calmodulin in Insulin Secretion

Much of the work implicating calmodulin in insulin secretion has depended upon inhibition by phenothiazines, especially trifluoperazine. This drug has been shown to be a potent inhibitor of glucose-stimulated insulin release in isolated rat islet cells [16, 19–23]. There is evidence also that the relative potencies of a variety of phenothiazines in inhibiting glucose- stimulated insulin release are parallel to their potency in inhibiting the activation of calmodulin-dependent phosphodiesterase [24]. Sulphoxide derivatives of the parent phenothiazines show little inhibition of calmodulin-dependent phosphodiesterase and do not inhibit insulin secretion. Furthermore, phenothiazines not only inhibit glucose-stimulated insulin release but also that induced by a number of other secretagogues, such as L-leucine and tolbutamide [22–24].

An unresolved question is whether the inhibitory effects of phenothiazines on insulin secretion can be entirely attributable to their binding to intracellular calmodulin. These drugs have other effects which might influence hormone secretion. Paradoxically, for example, at doses of trifluoperazine higher than 50 μmol/l there can be stimulation of hormone release at low glucose concentrations [20, 21]. It has been reported that 100 °mmol/l trifluoperazine can

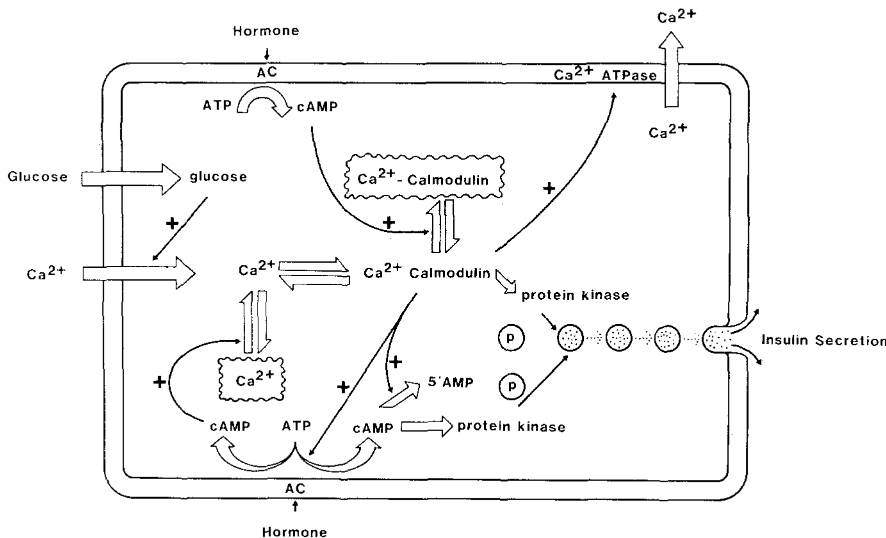

Fig. 1. A possible mode of action for calmodulin in insulin secretion. The possible biochemical events involved in the secretion of insulin where calmodulin may exert an effect are shown. The enclosed Ca^{2+}-calmodulin and Ca^{2+} represent that which is not free in cytosol but bound to membranes or within organelles.
AC: Adenylate cyclase; P: phosphorylation of protein; +: stimulation; for example Ca^{2+}-calmodulin can stimulate adenylate cyclase

cause slight stimulation of insulin release under basal conditions [22] and we ourselves have observed marked release of prolactin by isolated pituitary cells under similar conditions. There seems little doubt that this is due to a toxic effect of trifluoperazine since many cells are unable to exclude Trypan Blue at these high concentrations (unpublished observations).

There is some inconclusive evidence that phenothiazines can affect membranes. Over incubation periods longer than 60 min, significant inhibition of net calcium uptake into isolated islets can follow exposure to trifluoperazine [20, 22]; yet shorter incubation periods with this phenothiazine, which are equally inhibitory in terms of basal and stimulated insulin release, have no effect on net calcium uptake [19, 22]. Calcium fluxes across membranes may also be affected by trifluoperazine inhibiting a calmodulin-activated membrane bound ATPase [25].

Unfortunately, measurements of changes in calmodulin *concentration* using newly developed immunoassays [26] for the protein seem unlikely to clarify the role of calmodulin in stimulus-secretion coupling since available evidence is against changes in calmodulin mass in the cell but favours relocation or alteration in activity as the means by which its effects are exerted [27–29]. Clearly assays for intracellular activity and specific attempts at subcellular localisation under basal and stimulated conditions are essential to irrefutably establish the role of calmodulin in hormone secretion. Nevertheless, the absence of any effect of inhibitory doses of phenothiazines on glucose utilisation [16, 22] and the fact that the characteristics of inhibition of insulin secretion by phenothiazines are very closely related to the effects of the drugs on calmodulin activated phosphodiesterase [24] strongly suggests an involvement of calmodulin in insulin secretion.

Possible Mode of Action of Calmodulin in Insulin Secretion (Fig. 1)

Cyclic Nucleotides and Redistribution of Ca^{2+}-Calmodulin

There is now considerable evidence that calmodulin has effects on cyclic nucleotide synthesis and degradation in a variety of cell systems [12]. It seems possible therefore that the effects of calmodulin on insulin secretion could be related to some interaction with adenylate or guanylate cyclase or with the corresponding phosphodiesterase. Indeed, a calmodulin activated adenylate cyclase has been found in pancreatic islets [30]. However, no inhibition of cyclic AMP accumulation has been found in isolated islets under marked conditions of impaired insulin secretion following exposure to trifluoperazine [21, 22].

Moreover, using a Syrian hamster insulinoma model, glucagon stimulated insulin release, associated with elevation of cyclic AMP levels, was not inhibited by trifluoperazine [19]. In addition trifluoperazine had no effect on insulin secretion provoked by the phosphodiesterase inhibitors 3-isobutylmethylxanthine and theophylline which was accompanied by marked elevation of cyclic AMP concentration [22]. This evidence could be taken to indicate that cyclic AMP may be able to stimulate secretion in the absence of calmodulin-mediated events. However, it is possible that either trifluoperazine does not fully inhibit calmodulin or that cyclic AMP requires calcium ions but not calmodulin for its action. Despite the evidence against an interrelationship with cyclic AMP there is evidence that the cyclic nucleotide may trigger calmodulin redistribution within the cell, since phosphorylation of a membrane bound protein by cyclic AMP-dependent protein kinase caused release of cal-

modulin from the membrane into the cytosol in rat brain [27]. Redistribution of calmodulin has also been reported in studies of the effects of gonadotropin-releasing hormone on FSH and LH secretion in the pituitary; however in this case the redistribution was in the opposite direction, i.e. from cytosol to membrane [29].

Protein Phosphorylation

Recently, it has been suggested that calmodulin and cyclic AMP might trigger insulin release independently. Using a hamster islet cell line it has been shown that ^{32}P-phosphorylation of a 28K dalton protein occurs in association with the increased cyclic AMP levels and insulin secretion induced by glucagon [31]. In the same cell line, phosphorylation of another protein of molecular weight 98K dalton was enhanced by Ca^{2+}-calmodulin and diminished by trifluoperazine [19]. Under depolarising stimulatory conditions (50 mmol/l potassium) calcium-dependent phosphorylation of a further protein of molecular weight 60K was observed and both incorporation of ^{32}P and insulin release were inhibited by trifluoperazine at concentrations as low as 2.5 μmol/l; on the other hand glucagon acting via cyclic AMP had no effect on phosphorylation of this protein [32]. Furthermore, using a rat islet cell system other workers have also shown Ca^{2+}-calmodulin-dependent phosphorylation (inhibited by trifluoperazine) of a protein of molecular weight 55 K daltons [24]. The evidence suggests therefore that there are both cyclic AMP-dependent and Ca^{2+}-calmodulin-dependent (cyclic AMP-independent) protein kinases that can be activated separately under different stimulatory conditions. Nevertheless the possibility remains that the Ca^{2+}-calmodulin and cyclic AMP systems interact in some way either synergistically or consecutively.

Exocytosis, Microtubules, Microfilaments and Myosin Light Chain Phosphorylation

Insulin secretion in response to glucose is biphasic, with an initial rapid phase followed by a decline at around 5 min and then a secondary more prolonged phase; both of these phases of secretion are dependent on calcium ions [33]. The second phase probably involves synthesis of insulin de novo since it is blocked by inhibitors of protein synthesis [14]. The biosynthetic precursor of insulin is proinsulin [34]. Following its transport into the Golgi apparatus, this peptide is cleaved enzymatically to form insulin and C-peptide. The insulin is then packaged into secretory granules which move towards the plasma membrane [35]. The granule fuses with the plasma membrane and exocytotic discharge occurs [36]. A number of investigators have suggested that the microtubular and microfilament systems are implicated in the movement of granules in the B cell [37]. Glucose-induced insulin release is inhibited by substances which affect the polymerisation or stability of microtubules [38]. These microtubules might provide intracellular pathways for the secretory granules [39]. There is some evidence that calmodulin has a role in the process of assembly and disassembly of microtubules. Thus, it has been reported that the Ca^{2+}-calmodulin complex can both prevent the assembly and cause the depolymerisation of microtubules in vitro [40]. Glucose-induced insulin secretion has been shown to be enhanced by cytochalasin B, an agent which disrupts microfilaments. It has been proposed that the motile force necessary for movement of secretory granules may be provided by the microfilaments [41], which would presumably be mediated through the activation of myosin Mg^{2+}-ATPase by actin. This process is dependent on the phosphorylation of the light-chains of myosin, catalysed by myosin light chain kinase, an enzyme that is activated by calmodulin in the presence of calcium ions [42]. It appears therefore that both the intracellular pathways and the force required for the transport of the granules to the plasma membrane are controlled by the Ca^{2+}-calmodulin complex.

Conclusion

It is clear that calcium ions are of considerable importance as a second messenger in insulin secretion. There is increasing evidence that calmodulin, a ubiquitous intracellular regulatory protein that mediates calcium-dependent processes, has a fundamental role in stimulus-secretion coupling. Calmodulin is present in the B cell and the secretion of insulin is inhibited by phenothiazines which bind to and inhibit the action of calmodulin. The evidence strongly suggests that phenothiazines influence insulin secretion by their effect on calmodulin which probably mediates calcium-dependent insulin release. It seems likely that calmodulin acts at several points in stimulus-secretion coupling, influencing cyclic nucleotide metabolism, protein phosphorylation and exocytosis.

The discovery of calmodulin and the increasing clarification of its roles in cellular metabolism represent major steps towards our understanding of the mechanisms which influence the secretion of insulin.

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