Calmodulin and pancreatic B-cell function

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1. Introduction

In September–October 1979, two independent reports indicated the presence of the calcium-dependent regulatory protein, calmodulin, in rat pancreatic islet extracts[34, 39]. The reported values for the islet content in calmodulin averaged 0.11 and 0.13 pmoles per islet. In one of these studies, it was demonstrated that calmodulin binds to a membrane-rich particulate fraction prepared from a rat islet homogenate and causes activation of adenylate cyclase in the same subcellular material[39]. Both the specific binding of ^{125}I-labelled calmodulin and the activation of adenylate cyclase were Ca^{2+}-dependent processes[39]. In the other study, the allegedly specific inhibitor of calmodulin, trifluoperazine, was found to inhibit glucose-stimulated insulin release, whilst failing to affect the oxidation of glucose by rat islets[34]. These pilot observations thus documented the presence of calmodulin in islet cells and the capacity of calmodulin to affect the activity of a target enzymatic system in the islets, raising the view that calmodulin may be involved in stimulus-secretion coupling in the B-cell. Thereafter, from 1980 onwards, about 30 further publications and three reviews[28, 31, 36] were devoted to the same topic. The knowledge gained from these further studies indicates that calmodulin may affect the activity of several enzymes in the islets. However the precise role played by calmodulin in the secretory process remains to be fully elucidated.

In the present account, we will successively consider the following items: 1) the characterization and properties of calmodulin and calmodulin-binding proteins in insulin-producing cells, 2) the enzymes, present in these cells, of which the activity is influenced by calmodulin, and 3) the attempts made in intact islet cells to document a role for calmodulin in the process of insulin secretion.

2. Characterization and properties of calmodulin in insulin producing cells

As already mentioned in the introduction, the calmodulin content of rat pancreatic islets is close to 0.1 pmoles per islet (table). Assuming equal distribution in the intracellular space (about 2–3 nl/islet), this would correspond to a concentration close to 30–50 μM. Calmodulin was also identified in mouse islets[35]. Moreover, Hutton et al.[12] isolated calmodulin from a transplantable rat islet cell tumor. In the latter study, the tumor protein was compared in its physicochemical and biological properties with bovine brain and rat brain calmodulin. All preparations displayed comparable Ca^{2+}-dependent changes in native fluorescence and electrophoretic mobility, contained trimethyl-lysine, exhibited a blocked N-terminus, had identical amino-acid composition and molecular weight on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, yielded superimposable peptide maps after digestion with trypsin, papain or staphylococcus V-8 proteinase, demonstrated in equilibrium-dialysis experiments the binding of 4 g-atoms of calcium per mol of protein with equivalency between binding sites at a Kd close to 0.8 μM, were equipotent Ca^{2+}-dependent and trifluoperazine-sensitive activators of partially purified brain cyclic nucleotide phosphodiesterase, and all exhibited Ca^{2+}-dependent binding to troponin I, histone H2B and myelin basic protein[12]. Incidentally, Campillo and Ashcroft[3] indicated that calmodulin, prepared from bovine brain, may serve as a substrate for a protein carboxymethylase present in pancreatic islet homogenates. However, in intact islets, the inhibition of protein and phospholipid methylation by 3-deazaadenosine and DL-homocysteine, whilst causing a partial inhibition of glucose-stimulated insulin release, failed to affect the production of cyclic AMP by islets exposed to glucose in the absence or presence of the phosphodiesterase inhibitor 3-iso butyl-1-methylxanthine[1].

Calmodulin concentration in islets and insulin-producing cells

Tissue	Calmodulin content or concentration		Reference
Rat islets	0.11 pmoles/islet		34
Rat islets	0.13 pmoles/islet		39
Rat islets	0.09 pmoles/islet	0.14 pmoles/μg protein	35
Rat islets cytosol		0.25 pmoles/μg protein	19
Mouse islets	0.03 pmoles/islet	0.04 pmoles/μg protein	35
Hamster islets		0.10 pmoles/μg protein	23
RIN-m-5F cells		0.09 pmoles/μg protein	23
Rat islets cells tumor		*~0.13 pmoles/μg protein*	12

3. Calmodulin-binding proteins in insulin-producing cells

As indicated in the introduction of this review, the specific binding of ^{125}I-calmodulin to a subcellular particulate fraction derived from rat islets represents a Ca-dependent process[39]. Further work has contributed to the identification of calmodulin-binding proteins in insulin-producing cells. Thus, Nelson et al.[23] have identified calmodulin-binding proteins in a cloned rat insulinoma cell line, using a gel overlay technique. Ca-dependent binding of ^{125}I-calmodulin was observed to cytosolic proteins with apparent Mr = 125, 110, 56, 52 and 34 kDa. This binding was prevented in the presence of unlabelled calmodulin. The binding of ^{125}I-calmodulin to these proteins was also inhibited in a dose-dependent

manner by the anti-calmodulin drug W13. The latter drug also inhibited insulin release from the RIN cells, this inhibitory effect being reversible at least at low concentration (30 μM) of the drug. A less active analogue of the anti-calmodulin drug, W12, exerted little effect on ^{125}I-calmodulin binding and insulin release. These data suggest that W13 may inhibit insulin release by impairing the association of calmodulin to the calmodulin-binding proteins present in the islet cells[23].

4. Effect of calmodulin on islet enzymatic activities

a) Adenylate cyclase

Calmodulin activates adenylate cyclase in a 27,000 × g pellet of homogenized rat islets[39]. In these experiments, the islets were homogenized in the presence of EGTA and the pellet washed several times to remove endogenous calmodulin. Although Ca^{2+} itself (0.1–0.7 mM) caused a dose-related inhibition of adenylate cyclase activity, calmodulin (7 μM) increased by about 50% enzyme activity in the presence of 0.2 mM Ca^{2+}. No effect of calmodulin was observed at Ca^{2+} concentrations close to 10^{-7} M or less. Half maximal activation was observed at a Ca^{2+} concentration close to 10^{-5} M. In the presence of 0.2 mM Ca^{2+} the Ka for calmodulin was close to 0.1 μM[38]. Pharmacological agents considered as calmodulin antagonists (trifluoperazine and the trifluoromethylphenothiazine derivative of domperidone) caused a dose-related inhibition of the response to calmodulin but, in high concentrations, also inhibited basal adenylate cyclase activity. The nucleotide GTP slightly enhanced the stimulatory effect of calmodulin on adenylate cyclase[39]. Calmodulin also slightly increased NaF-stimulated enzyme activity[38].

The activation of adenylate cyclase by calmodulin was confirmed in rat islet particulate fraction by Sharp et al.[30] and Thams et al.[35]. In the first of these studies[30] the increment in reaction velocity attributable to calmodulin (6.6 μM) relative to basal value did not exceed 33% and could only be detected when 150 μM Ca^{2+} (or $CaCl_2$?) but not 200 or 250 μM Ca^{2+} (or $CaCl_2$?) was present in (or added to?) a buffer containing 200 μM EGTA. In the second study[35], the relative increment in reaction velocity attributable to calmodulin (1 μM) averaged 50% and was seen at ionized Ca^{2+} concentration in the 10 to 100 μM range but not at a lower Ca^{2+} level (1.0 μM). In this latter study, however, the stimulatory effect of calmodulin was restricted to the material derived from rat, but not from mouse islets, and this whether the enzymatic reaction was conducted in the presence or absence of GTP. In the rat system, the enhancing action of calmodulin was suppressed by trifluoperazine (50 μM) and displayed a Ka for calmodulin close to 0.1 μM[35].

The finding that Ca-calmodulin activates adenylate cyclase may have two important implications. First, it is known that endogenous cyclic AMP, although it fails to initiate insulin release, enhances secretion evoked by a variety of secretagogues. Since such secretagogues usually increase the cytosolic concentration of Ca^{2+}, the activation of adenylate cyclase by Ca-calmodulin would provide a device for amplification of the secretory response. Second, such an activation could also account for the fact that several secretagogues, which do not exert any direct effect upon adenylate cyclase in subcellular fractions, increase cyclic AMP production by intact islets. Such is the case, for instance, in glucose-stimulated islets. The view that the increase in cyclic AMP production evoked by glucose and other nutrient secretagogues in intact islets is mediated by Ca-calmodulin is supported by the finding that the nutrient-induced increment in cyclic AMP production is suppressed when the islets are deprived of extracellular calcium[34]. In this perspective, it should be duly underlined that the removal of extracellular calcium does not affect the production of cyclic AMP stimulated by secretagogues causing a primary activation of adenylate cyclase or primary inhibition of phosphodiesterase. For instance, in intact islets, the increase in cyclic AMP net production evoked by either forskolin or phosphodiesterase inhibitors is not impaired in the absence of extracellular calcium (Valverde et al.[37], and Valverde and Malaisse, unpublished observation).

b) Cyclic nucleotide phosphodiesterase

Two published reports deal with the possible effect of calmodulin upon cyclic nucleotide phosphodiesterase in rat pancreatic islets. Sugden and Ashcroft[33] first observed that, in the presence of Ca^{2+} (50 μM), calmodulin (0.12 μM) slightly enhanced, by no more than 13%, cyclic AMP phosphodiesterase activity measured at a relatively high concentration of the substrate (100 μM-cyclic AMP) in rat islet homogenates. The data obtained at increasing concentrations of the substrate suggested, however, that calmodulin affected solely the enzyme with a high affinity for cyclic AMP (Km: 6.2 μM). In the absence of added calmodulin, trifluoperazine inhibited cyclic AMP phophodiesterase activity, this effect being seen both in the absence or presence of Ca^{2+}. Moreover, at low substrate concentrations, the relative extent of inhibition appeared more marked in the absence of Ca^{2+} and calmodulin than in their presence. These findings suggest that, in this system, trifluoperazine did not act as a specific inhibitor of (endogenous or exogenous) calmodulin, and that a major fraction of the cyclic AMP phosphodiesterase activity is not sensitive to Ca^{2+}-calmodulin. Incidentally, calmodulin had no effect on cyclic GMP phosphodiesterase[33].

Lipson and Oldham[20] also observed that calmodulin (1.2 μM), in the presence of Ca^{2+} (150 μM) increases cyclic AMP phosphodiesterase activity in rat islet sonicates. Relative to the control value (Ca^{2+} 150 μM), the increase in reaction velocity averaged 16 and 13% at cyclic AMP concentrations of 10 and 500 nM, respectively. The effect of calmodulin, in the absence of Ca^{2+}, was not examined. Ca^{2+} alone augmented the enzyme activity (by 11–17%), possibly through activation by endogenous calmodulin.

These findings suggest that Ca-calmodulin may cause a modest activation of cyclic AMP phosphodiesterase in rat islets. To the extent that Ca-calmodulin also increases adenylate cyclase activity in the islet cells, the overall effect of this regulatory protein upon cyclic AMP metabolism could be viewed as an increase in the turnover rate of the cyclic nucleotide.

c) Ca-ATPase

In pancreatic islets like in other tissues, the extrusion of calcium from the cell into the extracellular fluid, against the prevailing electrochemical gradient, may be mediated either by a process of a Na–Ca countertransport or at the intervention of a Ca-activated ATPase located at the plasma membrane. Several studies were devoted to the possible effect of calmodulin upon the latter ATPase.

Owen et al.[25] reported that calmodulin causes a modest but significant inhibition of Ca-responsive ATPase in rat islet homogenates. The inhibitory effect of calmodulin faded out at high Ca^{2+} concentrations. In parallel experiments performed under identical conditions, calmodulin was found to activate kidney cortex Ca-ATPase and not to affect Mg-activated ATPase whether in the islet or kidney systems. A different picture emerges from the extensive study carried out by McDaniel and colleagues on the plasma membrane-associated Ca-activated islet ATPase.

Pershadsingh et al.[26] first observed that, in the presence of 0.13 μM Ca^{2+}, calmodulin (43 nM) stimulates ATP-dependent ^{45}Ca uptake by a plasma membrane-enriched fraction prepared from rat islets. Such an effect was not observed in an endoplasmic reticulum-enriched pellet[7]. In a further study, Kotagal et al.[16] tested the effect of calmodulin on ATPase activity in the same plasma membrane-enriched fraction. Calmodulin in concentrations up to 1.4 μM, failed to affect ATPase activity, whether in control membranes or those pretreated for 30 min with trifluoperazine (100 μM), when the Ca^{2+} concentration amounted to 1.1. μM and Mg^{2+} was used at its endogenous level (ca. 9 μM). It should be stressed that the latter concentration was precisely that used by the same authors to study the kinetics of the high affinity (Ca^{2+} + Mg^{2+}) ATPase in their first study[26]. In the second study, a stimulatory effect of calmodulin could only be detected when the Mg^{2+} concentration was raised to 200 μM and that of Ca^{2+} maintained at 1.1 μM[16]. However, no evidence was produced that, under these conditions, Ca^{2+} actually activated the enzyme. Moreover, the effect of calmodulin (0.2–1.4 μM) was most obvious in membranes pretreated with 100 μM trifluoperazine, in which case the control enzyme activity measured in the absence of calmodulin, was decreased by 89%[16]. Incidentally, in this second study, phenothiazines were shown to inhibit Ca^{2+} + Mg^{2+}-ATPase, but the drugs also inhibited Mg^{2+}-ATPase activity measured in the absence of Ca^{2+}[16]. In a further study conducted by the same group[6], at the high Mg^{2+} concentration (100 μM), Ca^{2+}-sensitive ATPase was apparently again demonstrated, but the corresponding absolute values for enzyme activity were not provided. It is troubling that the same data as those published earlier[16] – or otherwise, virtually identical data – were now described as representative of the calcium-stimulated component of ATPase activity[6]. Interestingly, calmodulin, in a concentration as low as 0.1 μM caused a more than 50% inhibition of the 'Ca-stimulated component of ATPase activity' measured in the endoplasmic reticulum-rich fractions. The latter finding is reminiscent of that reported by Owen et al.[25]. In a last study[15], an obvious stimulation of Ca^{2+} + Mg^{2+}-ATPase activity by calmodulin was seen in the presence of 0.9 μM Ca^{2+} and 9 μM Mg^{2+} when the EGTA concentration was decreased to 0.1 mM or when EGTA was omitted from the assay. However, under these conditions the control Ca^{2+}-responsive activity was reduced from about 20 nmoles/mg/min to about 1 nmole/mg/min. It is therefore fairly obvious that activation by calmodulin of the Ca^{2+}-responsive ATPase activity in the plasma membrane-rich fraction can only be detected under conditions in which such an activity is considerably reduced, e.g. as a result of pretreatment of the membranes with trifluoperazine or when the concentration of EGTA in the assay medium is considerably reduced.

These apparently complex, if not conflicting, results call for a cautious interpretation. The view that calmodulin may activate the Ca-responsive ATPase in the plasma membrane, as well documented in other tissues (e.g. in erythrocytes and kidney cortex), would indicate that Ca-calmodulin may play a role in facilitating the extrusion of Ca^{2+} from the cell. This, in turn, could represent a modality for restoration of the resting cytosolic Ca^{2+} activity in response to a prior stimulation of the cell with coinciding inflow of Ca^{2+} through gated Ca-channels. In this perspective the activation of the Ca-ATPase by Ca-calmodulin could participate in cyclic or rhythmic shifts between the stimulated (high cytosolic Ca^{2+} activity) and resting (low cytosolic Ca^{2+} activity) functional states of the islet cell.

d) Protein kinase

A number of observations indicate that islet cells display Ca-calmodulin-responsive protein kinase activity. Schubart et al.[27] using the cytosolic supernatant of hamster insulinoma cells, obtained after 90 min centrifugation at 48000 × g, identified a 98 kDa protein the phosphorylation of which was stimulated by Ca^{2+} (optimal Ca^{2+} concentration estimated at 0.12 μM) and, in the presence of Ca^{2+}, enhanced by calmodulin and inhibited by TFP (20–100 μM). In a subsequent work[29], these authors prelabelled the insulinoma cells with ^{32}Pi (60 min preincubation) and then incubated the cells for 10 min at low (4 mM) or high (50 mM) K^+ concentration. The depolarization of the cell at high K^+ concentration was associated with enhanced labelling of a Mr = 60 kDa protein. The latter effect was abolished in the absence of extracellular Ca^{2+} and in the presence of D600 or trifluoperazine (50 μM).

Ashcroft and his colleagues[8,10] have examined protein phosphorylation in rat islet homogenates exposed for 2 min to [γ^{-32}P]-ATP (20 μM). Ca^{2+} (40 μM) enhanced the phosphorylation of several proteins, especially that of a 53–55 kDa protein. In the presence of Ca^{2+}, the phosphorylation of this protein was further enhanced by calmodulin, the latter effect being antagonized by trifluoperazine (25–100 μM) and W7 (100 μM). Maximum phosphorylation occurred with 2 μM Ca^{2+} and 0.7 μM calmodulin. Brocklehurst and Hutton[2] also observed in the soluble protein fraction (40,000 × g, 60 min) from a rat islet cell tumor a 57 kDa protein, the phosphorylation of which proved dependent on Ca^{2+} (15 μM) and endogenous calmodulin. Likewise, in the cytosol of a

human insulinoma, Matsutani et al.[22] identified a 65 kDa and a 58 kDa protein, the phosphorylation of which was enhanced by calmodulin and inhibited by trifluoperazine. These authors also noted that calmodulin inhibits the phosphorylation of a 90 kDa protein. The latter effect was also antagonized by trifluoperazine, being possibly due to activation of a calmodulin-binding protein with phosphoprotein-phosphatase activity.

Further investigations aimed at the subcellular localization of the kinase(s) and the identification of the protein substrate(s). Landt et al.[19] prepared from rat islets a membrane particulate pellet (20,000 × g, 20 min). Simultaneous addition of Ca^{2+} (300 μM) and calmodulin (0.4 μM) enhanced phosphorylation of a single band with a Mr = 57 kDa. Half-maximal activation by calmodulin occurred at 0.36 μM. In the presence of Ca^{2+} and calmodulin, trifluoperazine inhibited the phosphorylation of this protein, half-maximal inhibition being reached at a concentration of trifluoperazine close to 40 μM. Curiously, these studies were performed in a 'heavy' particle fraction, although in the same report, the authors claimed that the calmodulin-activated protein kinase activity is confined primarily to a 'light' particle fraction (150,000 × g, 90 min). In a further study[4], by the same group, additional information was produced as follows. Using the microsomal or 'light' particle fraction, these authors identified two proteins (54 kDa and 57 kDa) as substrates for the Ca-calmodulin-sensitive kinase. Half-maximal activation occurred at 1.9–2.0 μM Ca^{2+} and 0.24–0.26 μM calmodulin, respectively. Brain tubulin added to the islet-cell microsomal fraction increased the labelling of the 57 kDa and 54 kDa protein bands. Pretreatment of the islet-cell microsomal fraction with anti-tubulin antibody prior to assay of protein kinase activity markedly decreased the subsequent Ca^{2+}- and calmodulin-dependent phosphorylation of both the 57 kDa and 54 kDa protein bands. These findings suggest that the calmodulin-dependent protein kinase may phosphorylate the α- and β-subunits of tubulin[4]. In the same work, intact islets were preincubated for 20 min with [^{32}P] Pi. The islets were then washed and incubated for 1–5 min at low (5 mM) or high (28 mM) glucose concentration, with or without theophylline (5 mM) and in the absence or presence of Ca^{2+}. Under these conditions, glucose was claimed to stimulate the phosphorylation of the 54 kDa protein, optimally over 3 min incubation. From the published figure, the glucose-induced increment did not exceed 23% of basal value[4]. Incidentally, the same group of investigators recently proposed that the Ca^{2+}- and calmodulin-dependent protein kinase activity in pancreatic islets is directly inhibited by alloxan[5].

Recently, Kowluru and McDonald[17], working with a rat islet cytosol preparation (105000 × g, 60 min), have also identified two phosphoproteins with Mr = 57 and 54 kDa respectively. The phosphorylation of the 57 kDa was preferentially stimulated by Ca^{2+} and calmodulin and that of the 54 kDa by cyclic AMP in the absence of Ca^{2+}. These two proteins differ from one another both by their pI on two-dimensional polyacrylamide gels, the 57 kDa protein being much more basic (pI 7.5–8.0) than the 54 kDa protein (pI 5.0–5.5) and by the Mr's of the native protein complexes as estimated by Sepharose 4B chromatography, the Mr of these complexes being close to 500 kDa for the complex yielding the 57 kDa protein and 180 kDa for that containing the 54 kDa protein. The latter protein could be labelled with tritiated azido-cyclic AMP by a photoaffinity technique. The authors concluded, from the difference in pI, that the 57 kDa protein could not be identified with a tubulin subunit[17].

Myosin light chains could also act as a substrate for a calmodulin-sensitive kinase in the islet cells. Thus pancreatic islet crude homogenates catalyze the incorporation of radioactivity from [γ-^{32}P]ATP in skeletal muscle myosin light chains[21]. The enzyme activity is enhanced in a dose-related manner by Ca^{2+} (0–100 μM) and calmodulin (0–10 nM) and inhibited by trifluoperazine (0–20 μM). After removal of endogenous calmodulin from the islet cytosol (105,000 × g × 60 min supernatant fraction), the Ka for Ca^{2+} and calmodulin were close to 10 μM and 2 nM, respectively, The islet enzyme activity could be bound to a calmodulin affinity column (Ca^{2+} present) and eluted from the column in the absence of Ca^{2+} and presence of EGTA. From these observations, it could be proposed that, when the binding sites of calmodulin are saturated with calcium, myosin light chain kinase could catalyze the phosphorylation of myosin which, in turn, should permit myosin to interact with actin to allow contraction of microfilaments in the B-cell[21].

5. Calmodulin and secretory granules

Watkins and Cooperstein[40] have reported that, in the presence of Ca^{2+} (100 μM), calmodulin (0.02–1.0 μM) causes a dose-related increase in the binding of inside-out plasma membrane vesicules to isolated secretory granules. This effect was suppressed in dose-related fashion by trifluoperazine (1–50 μM). Even in the absence of calmodulin, calcium (0.1–100 μM) stimulated the binding of the ^{125}I-plasma membranes to the secretion granule pellet. Such an effect, which was not observed with right-side-out vesicles, was equally sensitive to inhibition by trifluoperazine, a situation tentatively ascribed to contamination with endogenous calmodulin. It was concluded that Ca^{2+} in the presence of calmodulin modulates the interaction between the islet secretion granules and the cytoplasmic surface of the plasma membranes[40].

In a somewhat comparable perspective, Brockelhurst and Hutton[2] observed no effect of either Ca^{2+} or calmodulin on the phosphorylation of proteins in a purified insulin-granule fraction isolated from a transplantable rat islet cell tumor. However, after exposure to a soluble protein fraction (40,000 × g, 60 min) in the presence of Ca^{2+}, the secretory granules displayed Ca^{2+}-dependent phosphorylation of three proteins with Mr 100 kDa, 29 kDa and 10 kDa, respectively. Thus, components of the soluble fraction probably bound to intact granules in a Ca^{2+}-dependent fashion. Data were also obtained to suggest that the 100 kDa and 10 kDa proteins could dissociate from the granules after their phosphorylation[2].

6. Functional studies

Except for the study, already discussed, on the effect of Ca^{2+}-deprivation upon cyclic AMP production by intact islets, all investigations aiming at characterizing the possible role of calmodulin in the functional response of intact islet cells to suitable secretagogues were based on the use of drugs considered as calmodulin-antagonists, mainly trifluoperazine. As a rule, results comparable, though not identical, to those collected with trifluoperazine were obtained with chlorprothixene[28], prochloroperazine[8], 8-hydroxyprochloroperazine[8], 7-hydroxyprochloroperazine[8], the trifluoromethylphenothiazine derivative of domperidone[38], pimozide[11] and the naphtalenesulfonamide compounds W7, W12 and W13[23, 24, 28, 42], whereas trifluoperazine-5-oxide[8] or N-methyl-2(trifluoromethyl)phenothiazine[8] were unefficient. In the present report, the effect of these drugs on glucose metabolism, biosynthetic activity, cationic fluxes, cyclic AMP production and insulin release will be successively considered.

a) Glucose metabolism

Trifluoperazine (20–50 μM) fails to affect ^3H_2O and $^{14}CO_2$ production from islets exposed to [5-^{3}H] glucose or [U-^{14}C] glucose (10–20 mM), respectively[8, 11, 13, 34, 38]. However, at concentrations ranging from 100 to 300 μM, a dose-related inhibition of glucose oxidation was observed[38]. The drug W7 (50–100 μM) also fails to affect the oxidation of D-[U-^{14}C] glucose (10 mM)[24].

b) Proinsulin biosynthesis

Trifluoperazine (20 μM) fails to affect the glucose-stimulated incorporation of [4, 5-^{3}H] leucine in newly synthesized total islet protein or (pro)insulin[8]. Likewise, the drug W7, at concentrations up to 100 μM fails to affect (pro)insulin biosynthesis, inhibition of the latter process being only observed at a higher concentration of the drug (200 μM)[24]. Niki et al[24] claimed that W7 may affect the conversion of proinsulin to insulin, because the drug decreased the incorporation of ^{3}H-leucine in the insulin fraction over 120 min incubation. However, this effect was observed solely in the presence of 100 μM-W7, the ratio of tritiated insulin/(pro)insulin being unaffected at a lower (50 μM) or higher (200 μM) concentration of the drug[24].

c) Cationic fluxes

Trifluoperazine (25–50 μM) decreases ^{86}Rb efflux from glucose-deprived islets, whether in the absence or presence of extracellular Ca^{2+}[11]. At the same concentration, however, trifluoperazine does not abolish the capacity of glucose to decrease ^{86}Rb efflux. In addition, trifluoperazine (25 μM) inhibits K^+ influx as judged from the uptake of ^{86}Rb over 10 min incubation in the absence of glucose. The simultaneous inhibition of K^+ inflow and K^+ fractional outflow rate may account for the fact that trifluoperazine does not affect the net uptake of ^{86}Rb measured over 60 min incubation, whether in glucose-deprived or glucose-stimulated islets. These changes in K^+ handling may reflect a membrane action of the drug[11].

In the absence of glucose or at low glucose concentrations (1.7–2.8 mM), trifluoperazin (10–100 μM) fails to affect ^{45}Ca net uptake by islets over short incubation times (5–10 min), but inhibits ^{45}Ca net uptake over longer incubations (60–90 min)[11, 13, 38]. Trifluoperazine, in the 10–100 μM range, markedly inhibits glucose-stimulated ^{45}Ca net uptake, whether over short or prolonged incubations[11, 13, 38]. Pimozide (10 μM) also inhibits, although not severely, glucose-stimulated ^{45}Ca net uptake[11]. Over 5 min incubation, trifluoperazine (10 μM) also inhibits ^{45}Ca uptake stimulated by a high concentration of K^+, this inhibitory effect being more marked when the islets are preincubated for 30 min in the presence of trifluoperazine[13]. At variance with the latter observation, trifluoperazine (20 μM) was reported[27] not to affect ^{45}Ca net uptake by insulinoma cells exposed for 20 min, in the presence of 5.8 mM D-glucose, to either a high concentration of K^+ (40 mM) or ouabain (1.0 mM).

The view that trifluoperazine impairs the entry of Ca^{2+} into islet cells, as mediated by gated Ca^{2+}-channels, is supported by studies on the effect of the drug upon ^{45}Ca efflux from prelabelled and perifused islets. In this model, trifluoperazine (25 μM) exerts little or no effect upon ^{45}Ca efflux from islets perifused either in the absence of glucose, whether in the absence or presence of extracellular Ca^{2+}, or in the presence of glucose but absence of extracellular Ca^{2+}[11, 13, 38]. Under these conditions, trifluoperazine tends to decrease the fractional outflow rate of ^{45}Ca. Trifluoperazine (25 μM) fails to affect the capacity of glucose to decrease ^{45}Ca outflow from islets perifused in the absence of extracellular Ca^{2+}[11, 38]. At variance with these largely negative results, trifluoperazine (10–100 μM) obviously impairs the capacity of glucose to increase ^{45}Ca efflux at normal extracellular Ca^{2+} concentration[11, 13, 38]. This inhibitory effect of trifluoperazine is more marked when the islets are exposed to trifluoperazine prior to stimulation with glucose than when trifluoperazine and glucose are introduced simultaneously in the perifusion system[41]. Like its inhibitory effect upon insulin release the inhibitory effect of trifluoperazine on glucose-stimulated ^{45}Ca efflux from islets perifused at normal extracellular Ca^{2+} concentration represents a phenomenon which is not rapidly reversed upon removal of the drug from the perifusate[13, 38]. Trifluoperazine also inhibits the stimulation of ^{45}Ca efflux evoked in the presence of extracellular Ca^{2+}, by a rise in K^+ concentration from 6 to 24 mM[11]. Since the increase in ^{45}Ca efflux evoked by glucose or by a high level of K^+ in islets perifused at normal Ca^{2+} concentration reflects stimulation of Ca^{2+} entry into the islet cells, all these data suggest that trifluoperazine impairs the inflow of Ca^{2+} into the B-cell, as mediated by gated Ca-channels. It should be noted, however, that trifluoperazine also impairs the capacity of veratridine to stimulate ^{45}Ca efflux from islets perifused at low glucose concentration (2.8 mM) in the absence of extracellular Ca^{2+}[11]. The latter observation suggests the trifluoperazine may also impair the mobilization of Ca^{2+} from intracellular sequestration sites, as provoked by an increase in the intracellular Na^+ concentration. It is obviously a matter of speculation whether these effects of trifluoperazine upon ionic movements in the islet cells are mediated

through a specific calmodulin-antagonistic mechanism or by 'undiserable side-effects' of the drug. The fact that trifluoperazine, under suitable conditions, affects cationic fluxes in islets deprived of glucose or exposed to low concentrations of the sugar argues in favor of the latter interpretation.

d) Cyclic AMP production

Trifluoperazine, when used at concentrations sufficient to inhibit glucose-stimulated insulin release, only exerts marginal effects on cyclic AMP production by the islets. Thus, Karl[14] observed that trifluoperazine (10 μM) fails to affect the cyclic AMP content of islet exposed for 2 min to 27.5 mM glucose, whereas glucose-stimulated insulin release was virtually abolished by the same concentration of the calmodulin-antagonist. Comparable results were obtained by Valverde et al.[38] and Henquin[11] in islets stimulated for 10 min by glucose (10–16.7 mM), whether in the absence or presence of 3-isobutyl-1-methylxanthine. However, after 60 min incubation, and at high concentrations of trifluoperazine (25–50 μM), a partial or total suppression of the response to D-glucose was observed by these investigators[11, 38].

At the first glance, these results may appear incompatible with the view that Ca-calmodulin mediates the stimulant action of glucose upon cyclic AMP production. However, such an argument relies on the assumption that trifluoperazine indeed impairs calmodulin-sensitive events in intact cells. Yet, it is conceivable that the effect of trifluoperazine upon functional events in intact islet cells is unrelated to the calmodulin-antagonistic property of the drug. Moreover, trifluoperazine through a specific anticalmodulin action, may affect both the rate of cyclic AMP synthesis and breakdown, to the extent that adenylate cyclase and cyclic AMP phosphodiesterase are both activated by Ca-calmodulin.

e) Insulin release

In the absence of glucose or at low glucose concentrations, trifluoperazine (10–100 μM) tends to increase basal insulin output[18, 34, 38]. MacDonald and Kowluru have suggested that, at high concentrations, the calmodulin 'inhibitor' may act like calmodulin itself[21]. However, trifluoperazine inhibits glucose-stimulated insulin release. This was demonstrated in a number of models including incubated or perifused rat islets[11, 13, 18, 34, 38], the isolated perfused rat pancreas[42], a transplantable rat islet cell tumor[32], hamster insulinoma cells[27], a cloned rat insulinoma cell line[23] and even human pancreatic islets[9]. Complete suppression of insulin release is observed at high concentrations of the drug, but concentrations of trifluoperazine as low as 3 μM are sufficient to cause a partial reduction of the secretory response[38]. At this low concentration glucose-stimulated ^{45}Ca net uptake appears unaffected by trifluoperazine. The inhibitory action of trifluoperazine upon glucose-stimulated insulin release is an almost immediate, though progressive, but not rapidly reversible phenomenon[11, 38].

The inhibitory effect of trifluoperazine upon insulin release is not restricted to the secretory response to glucose. Thus trifluoperazine was also found to inhibit insulin secretion evoked by other nutrient secretagogues, such as D-glyceraldehyde, 2-ketoisocaproate or L-leucine[8, 11, 18]. Trifluoperazine also inhibits insulin release evoked, at low glucose concentrations (2.8–5.6 mM), by non-nutrient insulinotropic agents, such as glibenclamide or a high concentration of extracellular K^+ (24–40 mM)[8, 13]. Last, even in the absence of extracellular Ca^{2+}, trifluoperazine inhibits insulin release evoked by Ba^{2+}, veratridine or the association of glucose (16.7 mM) and ouabain[11, 13].

In certain circumstances, trifluoperazine fails to inhibit insulin release. Such is the case for the secretion of insulin evoked, in the presence of 5.6 mM D-glucose, by glucagon (3 μM) in insulinoma cells[27]. Trifluoperazine also does not inhibit, and may even enhance, insulin release evoked by phosphodiesterase inhibitors in islets incubated in the absence of glucose or at low glucose concentrations[11, 13]. The enhancing action of these phosphodiesterase inhibitors upon glucose-stimulated insulin release also appears to be unaffected by trifluoperazine[18, 38]. These findings indicate that trifluoperazine does not alter the insulinotropic response to endogenously formed cyclic AMP, as if the major effect of the latter nucleotide were to be located in the secretory sequence, at a site distal to the postulated calmodulin-sensitive step.

The data on insulin release so far reviewed are restricted to the effect of trifluoperazine. Comparable results were seen with other drugs. For instance, the naphtalenesulfonamide compound W7 (25–200 μM) also causes a dose-related, though only partial, inhibition of insulin release stimulated by D-glucose (10 or 20 mM). At the highest concentration (200 μM), W7 also enhances basal insulin output[24]. Likewise, W12 and W13 (30–100 μM) were found to inhibit insulin release in RIN-m-5F insulinoma cells exposed to 25 mM glucose; in this study, however, no control value was provided for basal insulin output[23]. Pimozide (1–10 μM) also causes a dose-related inhibition of insulin release evoked either by D-glucose (10 mM), whether in the absence or presence of theophylline (2 mM), by L-leucine or by Ba^{2+}[11]. The drug fails to affect the modest release of insulin evoked by a high concentration of theophylline (10 mM) in the absence of glucose[11].

Taken as a whole, these secretory data indicate that drugs known to bind calmodulin in tissue extracts exert dramatic effects on insulin release, even under conditions where they fail to affect early events in the secretory sequence, such as the metabolism of glucose or the inhibition of K^+ conductance. However, none of these functional data unambiguously establishes that the inhibitory effect of trifluoperazine and related drugs upon insulin release, as presumably resulting from interference with some distal step(s) in the secretory squence, is specifically related to inactivation of the normal response to endogenous Ca-calmodulin.

7. Conclusions

The evidence reviewed in this report unambiguously indicates the presence of calmodulin in insulin-producing cells and the activation by Ca-calmodulin of enzyme

systems in islet homogenates, with emphasis on adenylate cyclase and protein kinase. However, the extent to which calmodulin participates in the functional behavior of intact islet cells remains in our opinion, far from being elucidated. The functional data obtained with alleged specific inhibitors of calmodulin should be considered with due reservation. Indeed, there are several indications arguing against such a specificity, even in experiments performed in islet homogenates. In this perspective it would appear safer to assess the role of calmodulin in intact cells by comparing functional variables obtained in islets incubated in the absence or presence of extracellular calcium. However, this approach is obviously far from optimal, since the absence of extracellular calcium may affect functional events, e.g. insulin release, independently of the suppression of the response to Ca-calmodulin. With these considerations in mind, there is little risk to conclude that the participation of calmodulin in stimulus-secretion coupling remains a question open for further investigations.

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The role of cyclic AMP in insulin release

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Key words. Pancreas; cyclic AMP, role of; insulin release.

1. Introduction

The idea that adenosine-3′,5′-cyclic monophosphate (cyclic AMP) may act in the pancreatic B-cell as a second messenger was probably first raised when Samols et al.[72] reported that glucagon stimulates insulin release in man. The inhibitory effect of catecholamines on insulin secretion, first described by Coore and Randle[17], was also soon considered within the framework of this idea. Cerasi and Luft[11] even postulated that glucose, in addition to serving as a metabolizable substrate, acted in the B-cell on a specific membrane receptor leading to activation of adenylate cyclase, increased cyclic AMP production and stimulation of insulin release. In the latter model, cyclic AMP was considered to represent the signal for insulin release, whereas the metabolism of glucose would merely modulate this signal function, for example by increasing ATP availability to adenylate cyclase. At variance with the latter view, we had defended the concept that cyclic AMP should not be considered as a signal for insulin release but, instead, as a modulator of the metabolic and secretory response to nutrient secretagogues[53].

Almost 20 years have elapsed since cyclic AMP entered the field of insulin release, and more than a dozen years have passed since the contrasting views expressed above were introduced in relevant textbooks. In retrospect, it appears that the role of cyclic AMP in insulin release merits reevaluation. In the present report, the enzymes involved in the regulation of cyclic AMP synthesis and breakdown, the target systems responsive to this nucleotide and the role of cyclic AMP in the regulation of insulin release will be successively taken into consideration.

2. Cyclic AMP synthesis and breakdown

In the islets like in other tissues, the cell content in cyclic AMP is thought to reflect the balance between its rate of synthesis, breakdown and release in the extracellular fluid.

a) Cyclic AMP synthesis: adenylate cyclase

As judged by cytochemical criteria, adenylate cyclase is localized in the B-cell exclusively and almost uniformly in the plasma membrane[34]. In a particulate fraction (12,000 × g × 20 min) derived from the islets, the enzyme displays a Km for ATP close to 0.07 mM, whether in the presence or absence of NaF (10 mM)[49]. The temperature dependency (Arrhenius' plot) yields an apparent energy activation of 8.0 and 18.4 kcal/mole in the absence and presence of NaF, respectively[44]. The basal value for adenylate cyclase, expressed per islet-equivalent, is much higher in islet crude homogenates than in a subcellular fraction[21]. This difference is not solely attributable to uncomplete recovery of the enzyme in the particulate fraction. It may be due, in part, to be presence in the cytosol of islet cells of an unidentified phosphocompound which doubled the activity of adenylate cyclase in a particulate fraction (27,000 × g × 20 min) derived from mouse islets[88]. This factor was found to be dialysable, resistant to heat, sensitive to charcoal treatment and alkaline phosphatase, insensitive to digestion with trypsin; and distinct from either GTP, NAD or phospho-enol-pyruvate.

Consideration on the modulation of adenylate cyclase activity in the acellular system will here be restricted to those findings which are most relevant to the overall theme of this review and are not examined in greater detail elsewhere in this report.

Ca^{2+} (0.1 to 0.7 mM or more) causes a dose-related inhibition of adenylate cyclase activity[94]. However, in the presence of Ca^{2+}, the enzyme is activated by calmodulin[80,94]. At a fixed concentration of calmodulin, the threshold Ca^{2+} concentration for activation of the enzyme is close to 10^{-7} M, with an apparent Ka close to 10^{-5} M[94]. At a fixed concentration of Ca^{2+} (0.2 mM), the apparent Ka for calmodulin is close to 0.1 μM[93]. Curiously, Thams et al.[87] were unable to detect activation of adenylate cyclase by Ca-calmodulin in a mouse, as distinct from rat, islet particulate fraction.

Most investigators failed to detect any direct effect of