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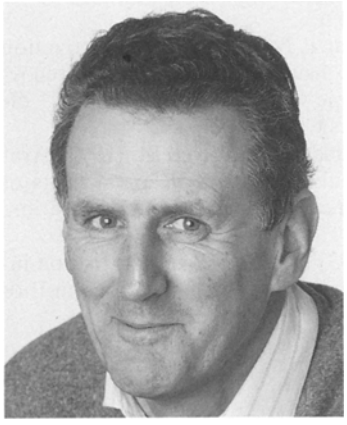

Simon L. Howell completed a Ph. D. on the regulation of insulin secretion under the supervision of Keith Taylor in 1967, and has sustained an interest in this topic ever since. Following a postdoctoral post in the laboratory of Paul Lacy in Washington University, Saint Louis, USA Dr. Howell joined Dr. Taylor's laboratory at the University of Sussex as a postdoctoral fellow before moving to a University Lecturership in London University in 1977. He was appointed Professor of Endocrine Physiology at King's College London in 1985, and has been Head of the Biomedical Sciences Division at King's College since 1988.

Regulation of insulin secretion: the role of second messengers

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Summary This review summarises briefly studies performed in the last 5–6 years concerning the role of second messengers in the regulation of insulin secretion, using intact and electrically permeabilized rat islets of Langerhans. It is concluded that cyclic AMP (through protein kinase A), calcium (through calcium-calmodulin dependent protein kinases) and diacylglycerol (through protein kinase C) may be important second messengers in modulating the effects of specific secretagogues on insulin release. However, recent studies strongly suggest that neither protein kinase A nor protein kinase C are directly involved in the regulation of insulin secretion by glucose. The possible involvement of other second messengers, nitric oxide and arachidonic acid, in the regulation of secretion is also briefly reviewed. [Diabetologia (1994) 37 [Suppl 2]: S30–S35]

Key words Islets of Langerhans, insulin secretion, protein kinase A, cyclic AMP, calcium-calmodulin, protein kinase C, arachidonic acid, nitric oxide.

We have attempted to evaluate the role of various second messenger pathways in the regulation of insulin secretion (Fig. 1). All of the work described here involved the use of rat islets of Langerhans, isolated by collagenase digestion; these 'normal' beta cells were used as a model in preference to the insulin secreting tumoural cell lines which have been used in many laboratories. The use of 'normal' islets has some disadvantages since only small amounts of tissue are available in relation to the cell lines, and they represent a heterogeneous cell population – only 70 % of the endocrine cell population being beta-cells. On the other hand it is clear that tumoural insulin secreting cell lines have characteristics which are significantly different from normal beta cells – not least in respect of their poor response to glucose stimulation and the lability of their metabolic characteristics according to passage number.

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Abbreviations: PKA, Protein kinase A; PKC, protein kinase C; PMA, phorbol myristate acetate.

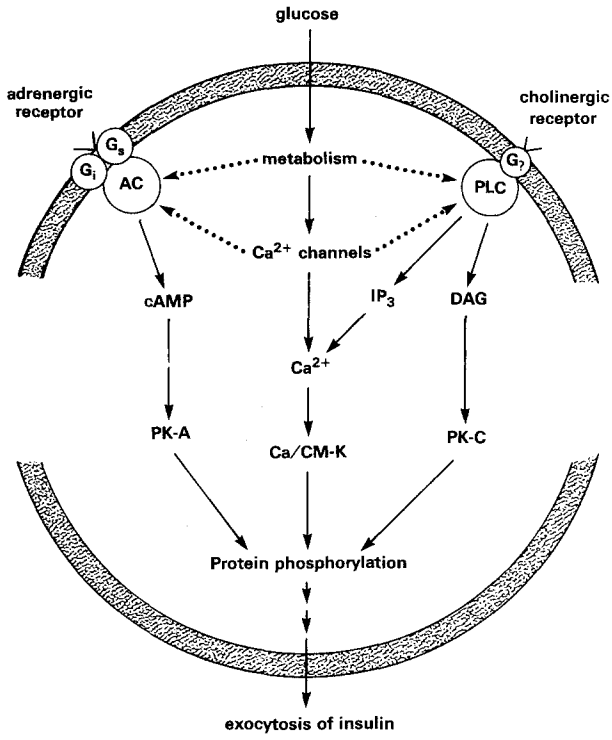

Fig. 1. Intracellular signalling pathways in the pancreatic beta cell. Glucose and agonists which act at cell surface receptors regulate insulin secretion by modulating the concentrations of intracellular second messengers which mediate their effects, in large, by affecting the activities of regulated protein kinases which phosphorylate protein substrates on serine and/or threonine residues

The techniques used in these studies were for the most part standard biochemical methods, with the exception of an electrical permeabilization technique, which we first adapted for use with intact islets (as distinct from individual cells in other tissues) in 1982 [1].

Electrical permeabilization has been used widely to study stimulus-response coupling mechanisms in a variety of secretory cell types [2]. This approach has substantial advantages over alternative detergent-based procedures in that for physical reasons only the plasma (cell) membrane is permeabilized [2], and all of the cells within the islet appear to be permeabilized to a similar extent (Fig. 2) [3]. Once permeabilized, the pores remain stable for a substantial period (several hours), in contrast to erythrocytes which reseal rapidly after application of similar techniques. Changes to the conditions of permeabilization, such as voltage or composition of permeabilization buffer, and use of alternative permeabilization procedures such as 'Transport' [4] have not allowed definition of treatment conditions in which such resealing of beta cells occurs.

The main advantage of use of permeabilized islets is an ability to alter the intracellular environment directly and rapidly by changing the composition of the incubation or perifusion medium. This has enabled direct control of, for instance, intracellular calcium, ATP, or cyclic nucleotide concentrations and determination of their effects on rates of insulin secretion [5, 6]. Secondly, it has been possible to see effects of changes in intracellular composition on the rates of proinsulin biosynthesis and processing [7]. On the other hand, electrically permeabilized cells have the disadvantage that they no longer respond to glucose stimulation of insulin secretion, for at least two reasons: (a) that they no longer fully metabolize glucose, possibly because of loss of low molecular weight cofactors in glucose metabolism; (b) the cells are fully depolarized because the intracellular and extracellular ionic environments are the same. This lack of glucose response is therefore not surprising. Secretion from the permeabilized cells is dependent on ATP and is temperature sensitive [5], and is stimulated by increasing calcium concentration in a range 50 nmol/l to 10 μmol/l [1, 5]. This is believed, from direct measurements of calcium concentration, to be within the range of calcium concentrations which exist in resting and stimulated beta cells. With the exception of their glucose responsiveness therefore, the electrically permeabilized beta cell provides a useful model for studying insulin secretion, and has been widely adopted for this purpose. A summary is presented of the use in our laboratory of intact and permeabilized rat islets in evaluating the role of second messengers in the regulation of secretion.

Cyclic AMP induced secretion

It has long been known that stimulants of adenylate cyclase such as forskolin, inhibitors of cyclic nucleotide phosphodiesterase such as isobutyl methylxanthine, or membrane-permeant analogues of cyclic AMP such as 8-bromo cyclic AMP can, in the presence of stimulatory glucose concentrations, potentiate insulin secretion [8]. In addition it is clear that exposure of islets to stimulatory concentrations of glucose results, among other changes, in increases in intracellular concentrations of cyclic AMP [9], although the majority of available evidence has suggested that these two are not directly linked. This question was addressed directly by the use of an isomer of adenosine 3'5' cyclic phosphorothioate (Rp-cAMP-S) the Rp form of which is a specific inhibitor of cyclic AMP dependent protein kinase (protein kinase A, PKA) [10]. Rp-cAMP-S, used at concentrations which in other systems have been shown to competitively inhibit PKA, was found to inhibit very effectively both forskolin-stimulated insulin secretion from intact islets, and 8-bromo cyclic AMP induced secretion from permeabilized islets [11]. However, it had no effect on calcium-induced secretion from permeabilized islets. In parallel experiments it was shown that Rp-cAMP-S inhibited the cyclic

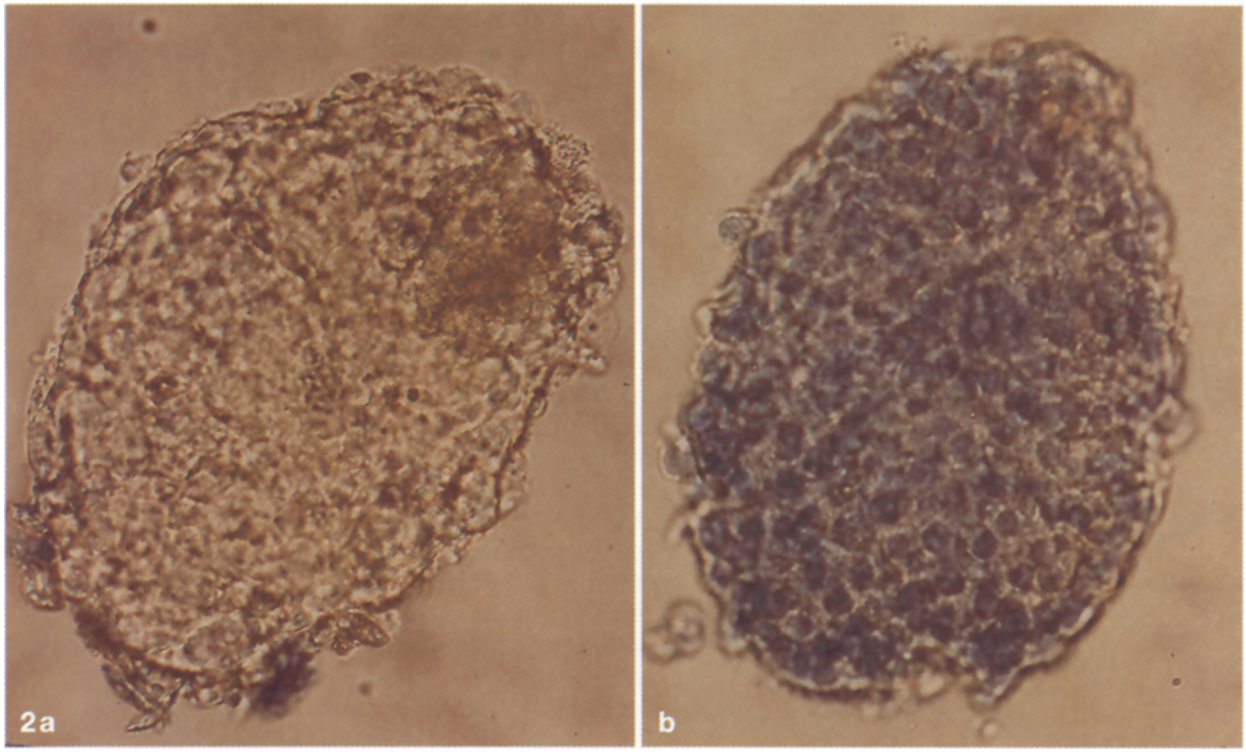

Fig. 2 a, b. Electrical permeabilization of islets. Micrographs of an intact (left) and an electrically-permeabilized (right) rat islet after exposure to trypan blue dye. Trypan blue does not enter intact cells but readily crosses permeabilized plasma membranes, staining the cells blue. Note that few cells in the intact islet have taken up the dye, whereas the cells in the electrically permeabilized islet are stained blue. Each islet is approximately 200 μm in diameter

AMP-induced phosphorylation of peptides in the range 20–90 kDa, without any effect on calcium-induced phosphorylation in similar conditions. In contrast glucose-stimulated insulin secretion from intact islets was not significantly affected by Rp-cAMP-S, suggesting that elevation of intracellular cyclic AMP concentration is not directly involved in glucose-stimulated insulin secretion [11]. Recent work using PKI-(6–22), a pseudo-substrate peptide inhibitor of PKA which potently inhibits cyclic AMP-, but not calcium-, induced secretion from permeabilized islets [12] has confirmed the essential role of phosphorylation in the action of cyclic AMP in beta cells. Lack of permeability of this peptide inhibitor in intact cells means that its effect on glucose-induced secretion cannot be tested directly.

Calcium-induced secretion

The role of calcium in the regulation of insulin secretion has been well recognised from early observations of the effects of omission of calcium from in vitro incubation media, and of the use of blockers of calcium channels, both of which inhibit insulin secretion [13]. More recently, increases in beta cell calcium concentrations have been measured directly [14–16], and increases in calcium concentration have been shown to be important determinants of insulin secretion from permeabilized beta cells [5]. In contrast to cyclic AMP-induced stimulation of secretion from permeabilized cells (which persists for at least 60 min of continuing stimulation), calcium-induced secretion in these conditions has a duration of only 15–20 min despite the continuing presence of the stimulus. Further experiments have been undertaken to determine why this should be the case, using perifused electrically permeabilized islets.

Restimulation of permeabilized islets which had already produced a substantial (15–20 min) response to calcium stimulation, after a delay of 10–20 min, did not reproduce this initial stimulatory response. Following this initial calcium response and release, the calcium insensitive (calcium-blind) cells were still able to respond to other stimuli such as cyclic AMP or phorbol myristate acetate (PMA) [3]. They were also able to release their single calcium response after a delay of up to 60 min, suggesting that this 'calcium-blindness' was not a result of loss of some component which is necessary for calcium-induced secretion, nor of diminishing viability of the cells. This calcium-insensitivity is mirrored in a reduction of phosphorylation of calcium-calmodulin dependent substrates [3].

This finding of 'calcium-blindness' following an initial stimulation was unexpected and of interest in a

Table 1. Summary of the involvement of protein kinases A, B and C in islet secretory responses

	Cyclic AMP	Ca^{2+}	PMA
Protein phosphorylation	Rapid, within 1 min	Rapid, within 1 min	Slow, within 15 min
Insulin secretion	Rapid, sustained increase	Rapid, transient increase	Slow, sustained increase
Effect of kinase inhibitors	Inhibit cAMP-, but not glucose-stimulated secretion	Inhibit glucose-stimulated secretion	Abolish PMA-stimulated secretion; reduce glucose-stimulated secretion
Effect of kinase down-regulation	Not possible	Selectively inhibits secretion in response to Ca^{2+}	Inhibits PMA-, but not glucose-stimulated secretion

Information obtained from [3, 5, 6, 11, 18, 19, 23, 24]. The kinase inhibitors used were Rp-cAMP-S (500 μmol/l), trifluoperazine (100 μmol/l) and staurosporine (50 nmol/l)

number of respects. For instance, it provides a possible rationale for the oscillations in intracellular calcium concentration which are observed in beta cells following exposure to glucose (see article by Hellman this issue) since the cells would clearly become calcium-insensitive during continuous prolonged exposure to high calcium concentrations. For technical reasons it is difficult to mimic in perifused permeabilized islets, oscillations of the duration observed in calcium measurement experiments. Secondly, these results shed further light on the question of whether PMA and cyclic AMP act by sensitizing the secretory mechanism of the beta cell to calcium [5, 6], or whether they can exert effects independently of activation of the calcium-sensitive secretory pathway. The fact that PMA and cyclic AMP are effective secretagogues in the calcium insensitive islets obviously suggests that these agents act, at least in part, by mechanisms other than increasing the sensitivity of the cells to calcium [3].

Protein kinase C induced secretion

Of the 9 sub-types of calcium phospholipid dependent protein kinase (PKC) now identified, at least two phorbol ester-sensitive isoforms (α, β) are known to be present in islets of Langerhans (see article by Ashcroft in this issue). Diacylglycerol, the physiological activator of PKC, increases in concentration in islets following exposure to carbachol (a stimulant of insulin secretion), and to a lesser extent to glucose [17]. It is therefore essential to assess the role of PKC activation in the regulation of insulin secretion in some detail. This was accomplished in part by the use of islets which are depleted of ~95 % of their PKC activity following culture for 24 h with PMA. This prolonged exposure effects the proteolytic degradation of the enzyme, so that the beta cells subsequently no longer respond to PMA, although islets cultured in the same concentrations of the inactive analogue 4αPMA will still respond normally to further PMA stimulation [18, 19]. These PKC-depleted islets were used to evaluate the role of this enzyme in secretory responses to a variety of stimuli. In brief, the secretory responses to cholecystokinin appeared to be wholly dependent on PKC stimulation, there being no response in the PKC-depleted islets [20]. Carbachol evoked an undiminished initial secretory response in PKC-depleted islets but the subsequent prolonged response was significantly diminished [21]. This is consistent with evidence of factors other than PKC, in particular calcium, in the secretory responses to this stimulus [22]. Finally, the characteristic biphasic insulin secretory response to glucose is unchanged in PKC-depleted islets [21], strongly suggesting that despite the increase in diacylglycerol synthesis observed on exposure of isolated islets to glucose, this does not provide an important signalling pathway for this secretagogue. However, our observations that inhibition of PKC activity with either non-selective (staurosporine) or fairly selective (Ro 31–8220) PKC inhibitors results in a reduction in glucose-stimulated insulin secretion [23, 24] might indicate that beta-cell phorbol ester-insensitive isoforms of PKC (ζ, ι), which would not be degraded on prolonged exposure to PMA, contribute to the secretory response to glucose.

Interestingly, noradrenaline, while inhibiting insulin secretion by mechanisms which have been considered to be protein kinase C independent, has a diminished effect on glucose stimulated insulin secretion in the presence of PKC activation [25]. One possible explanation, that PKC activation alleviates this inhibition by enhancing levels of cyclic AMP within the islets, has been eliminated in our experiments [25], and other possible explanations must now be considered.

The characteristics of secretory responses to cyclic AMP, calcium and PMA in permeabilized islets in the presence of substimulatory (50 nmol/l) Ca^{2+}, their time course of protein phosphorylation and effects of down-regulation are summarized in Table 1.

Other signalling pathways

Nitric oxide

There has been considerable interest in the possible role of nitric oxide as a signalling molecule in a wide

Table 2. Molecular weights of some endogenous substrates for islet protein kinases

Kinase	Molecular weight (kDa)																			
PKA	15	16				23	25	30				53			57	60			90	
CAMK					20						48	53		54	57	60				100
PKC				17	20			30		38								80		

The substrates indicated for each protein kinase have been reported by at least two laboratories. Note that each kinase phosphorylates a range of substrates of varying molecular weights and that no one substrate has been identified for all three kinases. CAMK, Ca^{2+}/Calmodulin-dependent protein kinase

variety of tissues, and it is clear that nitric oxide can be generated within the endocrine cells of both islets of Langerhans and insulin secreting cell lines. Nitric oxide synthase, the enzyme responsible for the formation of nitric oxide from arginine, can be activated by calcium and provides a possible candidate as a regulator of insulin secretion. A series of experiments was therefore performed to investigate this possibility including (i) direct measurement of nitric oxide generation in response to insulin secretagogues, (ii) testing effects of inhibitors of nitric oxide synthesis on insulin biosynthesis and secretion, (iii) measuring changes in islet cyclic GMP concentrations, (iv) testing effects of exogenous cyclic GMP on insulin secretion. The results of these four lines of investigation were consistent in indicating no evidence for a role of nitric oxide as a second messenger in initiating secretory responses to common secretagogues [26], although they do not detract from the potentially important role of nitric oxide in beta cell destruction during the development of insulin-dependent diabetes (see article by Nerup in this issue).

Arachidonic acid

Arachidonic acid is present in islets and can be generated from phosphatidylcholine via phospholipase A_2 and from diacylglycerol via diacylglycerol lipase. After synthesis it can be metabolized to a whole series of biologically active molecules via both cyclooxygenase and lipoxygenase pathways. A possible role of arachidonic acid or its metabolites on insulin secretion has been postulated, and reviewed in detail [27–29]. More recently evidence has emerged which may suggest that arachidonic acid itself may play a direct role in secretion. Most of the signal transduction mechanisms outlined above can be eliminated from roles as second messengers in arachidonic acid induced secretion, which occurs in the absence of glucose [30], in contrast to agents acting through phospholipase C or adenylate cyclase activation, which require glucose to be present. Similarly, the effectiveness of arachidonic acid as a secretory stimulus in permeabilized islets argues against depolarization of beta cells as a mechanism of its action [29]. Calcium entry may be one possible mode of action, although in our experiments arachidonic acid was found to stimulate secretion in the absence of extracellular calcium, and in permeabilized islets at very low intracellular calcium concentrations (50 nmol/l) [30].

Recent experiments in permeabilized islets have shown that arachidonic acid can stimulate phosphorylation of a specific protein of ~18 kDa molecular weight which appears quite distinct from any known endogenous substrate for PKC [31]. Interestingly this phosphorylation is quite distinct from the effects of arachidonic acid on secretion, as indicated by the lack of effect of protein kinase inhibitors such as staurosporine which did not affect the arachidonic acid stimulation of insulin secretion despite effective inhibition of arachidonic acid-induced phosphorylation [32].

This, and other evidence, leads us to conclude that arachidonic acid itself may be acting as second messenger in beta cells, perhaps acting as a fusogen to promote membrane fusion/fission during exocytosis [29].

Second messengers and activation of the insulin secretory mechanism

Ten years ago it seemed reasonable to speculate that a single substrate (we speculated that myosin light chain is a candidate) might provide a single focus for the various signalling pathways of secretion, providing a simple link between the metabolic signals produced via second messengers and the physical process of exocytosis [33]. This view now appears unlikely to be correct, on the grounds that analysis of the endogenous substrates for protein kinases in islets has demonstrated substrates of a variety of molecular weights (Table 2), with no single molecular species clearly demonstrable as a common substrate for protein kinases A, B and C. It cannot, however, be excluded that such a substrate is not detectable by present methods, or that it turns over very rapidly rendering its identification in phosphorylation experiments very difficult. Analogy with other systems also suggests that the details of apparently simple pathways may in fact be complicated – for instance detailed studies of the actions of insulin mediated via IRS-1 [34] and of glycogen synthesis and degradation [35] reveal great complexity in what had originally been believed to be relatively simple pathways. At the same time, however, the study of organelle movement in various cells is now advancing rapidly, and

several new organelle 'motors' have been identified, and their molecular control studied in some detail [36]. It may be some time before we can fully understand the true complexity of the insulin secretory mechanism.

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