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COMMENTARY

REGULATION OF GLUCAGON RELEASE FROM PANCREATIC A-CELLS

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The hyperglycemic hormone glucagon is secreted from the A-cells of the endocrine pancreas. Due to the application of novel techniques, significant progress in our understanding of stimulus–secretion coupling in glucagon-secreting A-cells has been made during the last few years. First, with the aid of cell-sorting methods, it has been possible to perform biochemical experiments on pure A-cell preparations [1]. Second, the introduction of microfluorometric techniques has enabled measurements of the cytoplasmic free Ca^{2+} concentration in single A-cells [2, 3]. Finally, the patch-clamp technique has allowed recording of electrical activity and characterization of the participating ion currents in glucagon-secreting cells [4–6]. These new experimental data are reviewed in this commentary and an attempt is made to integrate them into a working hypothesis for the regulation of glucagon release.

Regulation of glucagon release by nutrients and hormones

In this section we summarize the actions of physiological modulators of glucagon secretion. It should be emphasized that the list is far from comprehensive and is restricted to the major regulators. For convenience, we discuss these regulators according to their capacity to inhibit or stimulate glucagon release.

Inhibitors. It is well established that glucose inhibits glucagon release [for review see Ref. 7]. The threshold for inhibition of glucagon release by glucose has been reported to be 2–3 mM, which is somewhat below that for stimulating insulin release [8]. Glucose uptake into purified A-cells is less pronounced than that in B-cells and is not stimulated by insulin. Furthermore, the rate of glucose metabolism is only about 15–20% of that observed in the B-cell [9]. The inhibitory effect of glucose on glucagon secretion is shared by other glucose metabolites and related sugars [7]. In general, the inhibitory capacity is determined by the ability of the cells to metabolize the sugar. This is consistent with the observation that glucose-induced inhibition of glucagon release can be counteracted by inhibitors of cellular metabolism such as 2,4-dinitrophenol [7] or anoxia [10]. In fact, the latter conditions result in pronounced *stimulation* of glucagon release. This effect may reflect a direct inhibitory influence of A-cell metabolism on glucagon release. However, insulin secretion is abolished under these experimental conditions, and it must therefore be considered whether the effect of metabolic poisoning is secondary to relief from a paracrine inhibition by the B-cell.

It is well established that somatostatin inhibits secretion of both insulin and glucagon from the endocrine pancreas. Somatostatin is produced and secreted by the pancreatic D-cells as two peptides containing 28 (S28) and 14 (S14) amino acids respectively [11]. Release of somatostatin generally occurs in parallel with that of insulin [12]. The mechanisms whereby somatostatin inhibits glucagon release are still unknown. Two main mechanisms can be proposed: (1) interference with second messenger systems and the exocytotic machinery; or (2) alterations of membrane ion conductances. These processes have been demonstrated to contribute to the inhibitory action of this hormone on insulin secretion [13, 14].

The pancreatic A-cell was formerly regarded as the prototype of an insulin-sensitive cell. However, the elegant studies of Pipeleers and co-workers [15, 16] on purified A-cells have demonstrated clearly that this is probably not the case. First, binding studies of radioactive insulin have shown that there are less than 400 insulin receptors per A-cell, a receptor density which is 50 to 500-fold lower than in classical insulin-sensitive cells [16]. Second, insulin is without effect on A-cell electrical activity [4] and glucagon release [15].

Stimulators. Catecholamines play an important role in those metabolic adjustments which increase the availability of glucose during stress. Apart from mobilization of hepatic glycogen, these mechanisms involve inhibition of insulin secretion and stimulation of glucagon release. Whereas the effect of catecholamines on the B-cells has been shown to be mediated by α_2-adrenoreceptors [17], the action on the A-cell involves activation of β-receptors [18]. The effects of β-adrenergic stimulation are believed to be mediated by increased cytoplasmic levels of cyclic AMP [18]. Accordingly, adrenaline-induced changes in glucagon secretion and in A-cell content of cyclic AMP show the same concentration dependence [18]. Moreover, agents stimulating

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adenylate cyclase, such as forskolin, mimic the action of adrenaline (see below).

The amino acids glutamine, alanine and arginine initiate glucagon secretion, with arginine being the most potent [15]. Other amino acids have either no, or only slight, stimulatory action on their own but are capable of potentiating secretion induced by another amino acid. This is supported by the observation that the addition of a mixture of 20 amino acids leads to a pronounced stimulation of glucagon release which exceeds that which would be expected if the effects were just additive [15]. These findings suggest that circulating amino acids play an important role for the regulation of glucagon release *in vivo*. Electrophysiological experiments have demonstrated that arginine increases the A-cell action potential frequency. The effect is characterized by a rapid onset, indicating that metabolic degradation of this amino acid is not required [4], and it has been suggested that this positively charged amino acid depolarizes the A-cell by its electrogenic entry [4].

Role of Ca^{2+} and A-cell electrical activity

Although earlier studies indicated that the A-cell secretory rate is inversely related to $[Ca^{2+}]_i$ [for review see Ref. 19], it is now generally accepted that initiation of glucagon release is preceded by an increase in $[Ca^{2+}]_i$. Evidence in support of this concept is provided by the observations that: (1) glucagon secretion induced by physiological secretagogues is dependent on the presence of extracellular Ca^{2+} [15]; (2) glucagon release can be stimulated by the Ca^{2+} ionophore A23187 [20]; (3) $^{45}Ca^{2+}$ fluxes in islets from streptozotocin-treated guinea pigs (which contain 60% A-cells), indicate that glucose-induced inhibition of glucagon secretion involves reduction of Ca^{2+} uptake [21]; (4) studies employing permeabilized cells show that glucagon secretion increases with the intracellular Ca^{2+} concentration [22]; (5) compounds which stimulate glucagon secretion increase the frequency of Ca^{2+}-dependent action potentials [4]; and (6) microfluorometric measurements using fura-2 demonstrate that experimental conditions known to stimulate glucagon secretion are associated with increased $[Ca^{2+}]_i$ [2, 3].

Patch-clamp experiments on isolated A-cells have shown that these cells produce spontaneous action potentials [4]. These action potentials are initiated from a membrane potential of about −65 mV and peak at +20 mV. Voltage-clamp measurements have demonstrated that the A-cell is equipped with voltage-dependent Na^+, Ca^{2+} and K^+ currents. Whereas activation of the inward Na^+ and Ca^{2+} currents underlies the depolarization of the action potential, the repolarization results from activation of the outward K^+ current [4]. Based on pharmacological and biophysical differences, it appears that the A-cell is equipped with two types of voltage-dependent Ca^{2+} channel [5], with properties reminiscent of those reported for L- and T-type Ca^{2+} channels in neurones [for terminology see Ref. 23]. The single-channel currents underlying the T- and L-type Ca^{2+} currents in the A-cell are shown in Fig. 1. Whereas openings of the T-type channel can be elicited at membrane potentials as negative as −60 mV, more positive voltages are required to activate L-type channel openings. The differences in the voltage dependence of activation suggests that the two Ca^{2+} currents may have different functions in the A-cell. The L-type Ca^{2+} current activates at membrane potentials corresponding to the rapid rising phase of the action potential, and it is therefore likely to participate in this process. The T-type Ca^{2+} channels open at membrane potentials close to the threshold for action potential initiation and may consequently play a role in the pacemaking of the A-cell. Such a concept is supported by the observation that spontaneous electrical activity cannot be maintained in a Ca^{2+}-free solution, although the Na^+ current is several-fold larger than the two Ca^{2+} currents taken together [4]. A function for a voltage-dependent current in the pacemaking of the A-cell would also explain why a small depolarization, such as that obtained by applying arginine, markedly increases the action potential frequency [4]. Conversely, it can be predicted that any agent or condition associated with the development of an outward hyperpolarizing current will markedly reduce electrical activity and inhibit glucagon secretion. However, the observation that glucose, whether in the presence or absence of insulin, fails to suppress the A-cell electrical activity [4] suggests that the sugar does not itself produce the activation of such a conductance and implies that inhibition of glucagon secretion involves other, more indirect, mechanisms.

Intracellular messenger systems

Agents which increase the intracellular cyclic AMP concentration stimulate glucagon secretion [18]. Cyclic AMP is able to mobilize Ca^{2+} from intracellular stores [3]. However, the resulting $[Ca^{2+}]_i$-transient is much shorter than the duration of the associated stimulation of glucagon secretion [3]. It has therefore been suggested that cAMP also acts by sensitizing the secretory machinery to Ca^{2+} [3]. Such a concept is consistent with the stimulatory effect of forskolin on glucagon secretion at low (0.1 μM) Ca^{2+} concentrations in permeabilized islets [22]. There is also some evidence that cyclic AMP may enhance Ca^{2+} influx through L-type Ca^{2+} channels [5].

Activation of protein kinase C by phorbol esters such as 12-*O*-tetradecanoyl-13-acetate (TPA) has been reported to augment glucagon secretion from both intact and permeabilized islets [20, 22]. A role for protein kinase C in arginine stimulation of glucagon secretion has also been postulated [20]. This idea is not necessarily in conflict with the observation that the stimulatory effect of arginine on A-cell electrical activity is characterized by a rapid onset [4]. Separate mechanisms may account for different phases of glucagon secretion. For example, activation of protein kinase C may be important for maintaining glucagon secretion during a long period of stimulation, while a more direct effect of arginine on A-cell electrical activity may account for the rapid response.

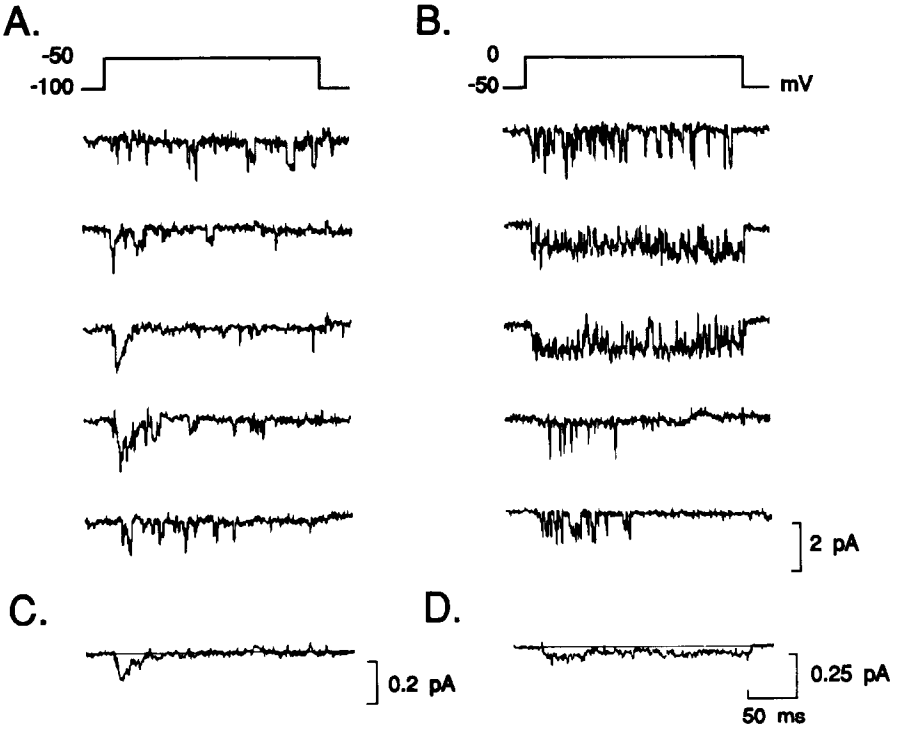

Fig. 1. Two types of Ca^{2+} channel in pancreatic A-cells. Ca^{2+} channel activity recorded from a cell-attached patch on a single guinea pig A-cell is shown. (A) Activity of the T-type Ca^{2+} channel seen during depolarizations to −50 mV from a holding potential of −100 mV. Note that the openings are concentrated at the beginning of the voltage pulses, with little activity remaining at the end of the depolarization. The amplitude of the single-channel events was ≈0.5 pA, from which a single-channel slope conductance of ≈5 pS can be estimated (assuming a reversal potential of +50 mV). (B) L-type Ca^{2+} channel activity recorded during depolarizations to 0 mV from a holding potential of −50 mV. Channel openings occur evenly throughout the voltage pulses. The amplitude was ≈1 pA, which corresponds to a single-channel conductance of ≈20 pS. (C) Associated mean T-type Ca^{2+} current for depolarizations to −50 mV. Note inactivating time course. (D) Associated mean L-type Ca^{2+} current for the depolarization to 0 mV. The current has a more sustained time course than that in C. The recording pipette contained 100 mM $BaCl_2$, 10 mM TEA and 5 mM HEPES [pH 7.4 with $Ba(OH)_2$]. The two different pulse protocols were used to permit selective activation of either T- or L-type Ca^{2+} channels.

Mechanisms of glucose inhibition of glucagon release

The glucose concentration experienced by the islet cells varies between 4 and 7 mM, these being the blood glucose concentrations observed in healthy individuals during fasting and subsequent to a glucose-rich meal respectively. Processes regulating insulin and glucagon secretion must operate within this concentration range to be of physiological importance. When considering the regulation of glucagon secretion *in vivo*, it should be emphasized that *in vitro* techniques generally destroy the normal organization of the pancreas and disrupt neural, circulatory and paracrine regulatory mechanisms. It is therefore possible that the glucose dependence of stimulation and inhibition of hormone release is different in the intact organ from that suggested by *in vitro* measurements.

Glucose has been suggested to inhibit glucagon secretion by stimulating the removal of Ca^{2+} from the cytoplasm, thus lowering $[Ca^{2+}]_i$ [2, 3]. Indeed, an elevation in the glucose concentration from 0 to 20 mM has been shown to result in a reduction of $[Ca^{2+}]_i$. However, it is unclear whether these effects can be extended to explain the situation *in vivo*; no data have yet been presented on the effects of varying the glucose concentration within a more physiological range.

It is well established that the endocrine part of the pancreas contains the inhibitory neurotransmitter γ-aminobutyric acid (GABA) at concentrations comparable to those encountered in the central nervous system [24]. GABA has been shown to co-localize with insulin in the β-cells [25], but the subcellular distribution is not known. Circumstantial evidence that GABA is present in the β-cell granules comes from the observation that glucose concomitantly increases the release of insulin and GABA [26]. Additionally, the islet content of GABA has been shown to decrease under a number of experimental conditions that are associated with stimulation of insulin secretion [27].

In nerve cells GABA reduces electrical excitability

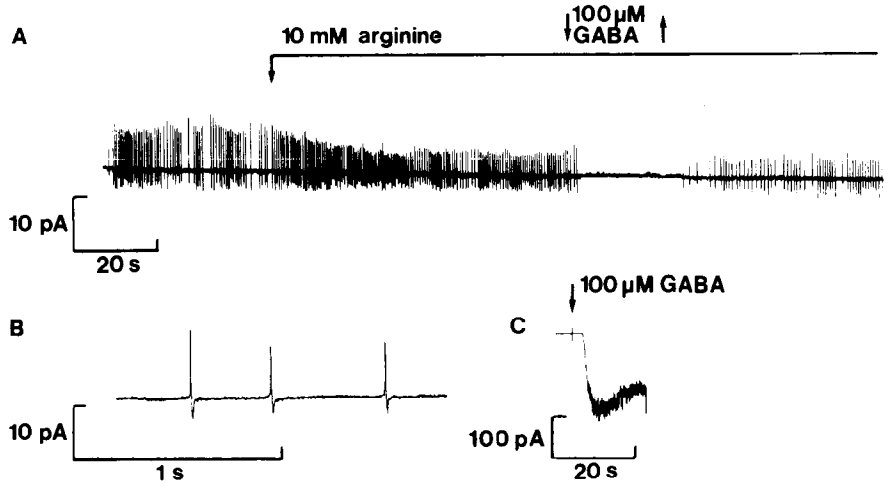

Fig. 2. (A) Electrical activity recorded from a cell-attached patch on an isolated guinea pig A-cell. The cell produces spontaneous action potentials under basal conditions. Addition of 10 mM arginine leads to increased spike frequency. The gradual reduction of spike amplitude can be explained in terms of voltage-dependent inactivation of the Na^+ current [4]. Addition of 100 μM GABA reversibly inhibits electrical activity. (B) Example of biphasic current deflections due to action potentials, displayed on an expanded time scale. The patch potential was 0 mV in A and B. (C) Whole-cell recording of membrane currents from same cell as in A and B. Addition of 100 μM GABA leads to the development of a large inward current when holding at −70 mV. With the solutions used, the Cl^- concentration is about the same on both sides of the membrane (≈150 mM). At negative membrane potentials, activation of the channel will, therefore, result in outward Cl^- flux, giving rise to inward currents.

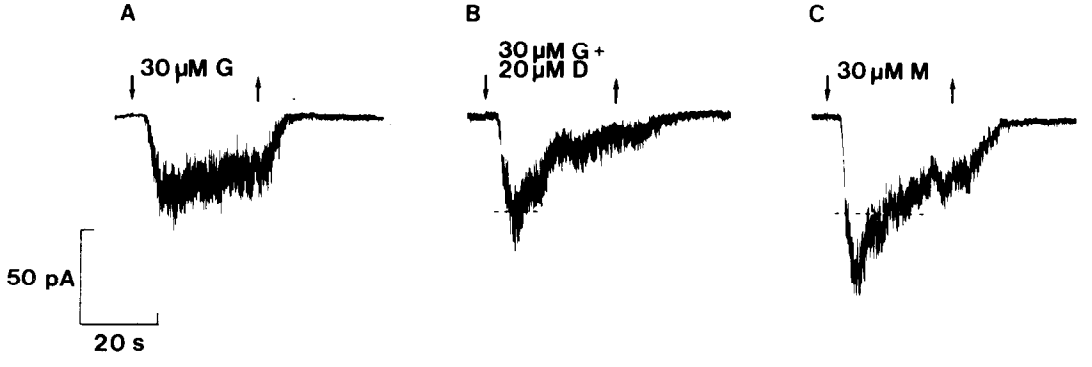

Fig. 3. Pharmacological properties of the GABA-activated Cl^- channel in a guinea pig A-cell. (A) Current evoked by 30 μM GABA (G). (B) Current evoked by 30 μM GABA in the presence of 20 μM diazepam [D; dissolved in dimethyl sulfoxide; final concentration: 0.1%]. Note the increased amplitude and rate of desensitization. (C) Current evoked after equimolar replacement of GABA with muscimol (M). Note the increased amplitude and rate of inactivation. The dashed lines in B and C indicate the control peak current (A). GABA-activated currents are inward because of the high intracellular Cl^- concentration.

by increasing the membrane Cl^- permeability. We have demonstrated recently that a similar mechanism is operational in the A-cell [6]. However, these experiments were performed using the whole-cell configuration of the patch-clamp technique. In this recording mode the intracellular Cl^- concentration is set by the pipette solution which dialyzes the cell interior. It is therefore important to demonstrate that GABA exerts an inhibitory action also on the electrical activity of intact A-cells. Figure 2A shows a recording of action potential activity from a cell-attached patch on an isolated A-cell. In this patch configuration, normal ion gradients are retained. The A-cell generates action potentials under basal conditions; these are seen as biphasic current deflections due to the capacitive properties of the patch membrane [for explanation see Ref. 28]. It is worth pointing out that the absence of any applied secretagogue can be regarded as an A-cell stimulus and that glucagon secretion is elevated under

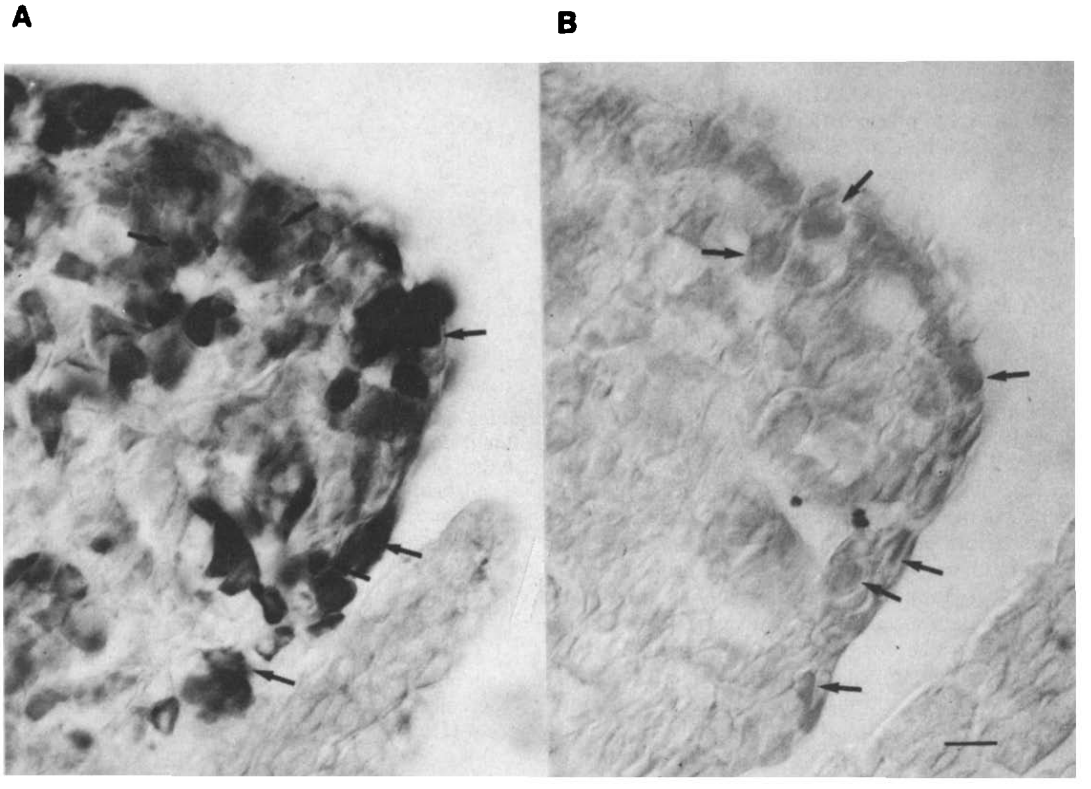

Fig. 4. (A) Section through a rat pancreatic islet stained with antibodies against glucagon. (B) A consecutive section stained with antibodies against the β-subunit of the $GABA_A$-receptor Cl^- channel complex. The sections were 30 μm thick. Note that the distribution of glucagon is similar to that of the $GABA_A$ receptor. The scale bar corresponds to 20 μm. The antigen–antibody complexes are visualized by the use of an indirect peroxidase-antiperoxidase (PAP) technique with 3,3′-diaminobenzidine as the chromogen.

this experimental condition. The action potential frequency increases when glucagon secretion is stimulated by 10 mM arginine. Addition of 100 μM GABA reversibly suppresses spiking activity, indicating that a hyperpolarizing current is activated by GABA. Indeed, a subsequent whole-cell recording confirmed that this cell possessed a large GABA-activated Cl^- current (Fig. 2C).

The magnitude of the GABA-activated Cl^- current depends on the concentration of GABA. Half-maximal activation is observed at ≈40 μM [6]. Taking the interstitial islet volume to be one-third [29] of the total islet water (1.6 nL; [30]), we estimate such a concentration will occur if 0.2% of the total B-cell content of GABA is released. This compares favorably to the percentage of insulin released per minute during stimulation with 20 mM glucose (Berggren P-O and Rorsman P, unpublished observations). These considerations indicate that physiologically active concentrations of GABA may develop within the islet during stimulation of insulin secretion.

At concentrations exceeding 30 μM the currents evoked by GABA decline spontaneously, despite the continued presence of the agonist, a process referred to as desensitization. This may explain the observation that glucose is a less potent inhibitor of glucagon secretion in static incubations than in the perifused rat islets or perfused pancreas [7]. In batch incubations, GABA is more likely to accumulate in the islet interstitium. The resulting desensitization of the GABA-activated Cl^- channels would make the A-cell less sensitive to newly released GABA.

Figure 3A shows GABA currents recorded in response to 30 μM GABA. Addition of a 20 μM concentration of the benzodiazepine diazepam increases the current responses and accelerates desensitization (Fig. 3B). Replacement of GABA with the $GABA_A$-receptor agonist muscimol produces an approximate doubling of the current and more rapid desensitization (Fig. 3C). The currents elicited by GABA in the A-cell could be blocked by a 25 μM concentration of either the GABA-receptor antagonist bicuculline or the channel blocker picrotoxin [6]. This pharmacological evidence suggests that the GABA-activated Cl^- channels in A-cells are of the $GABA_A$ type [for review see Ref. 31].

Histochemistry on intact pancreas using [^{3}H]GABA has suggested that the receptors are restricted to the islet periphery with little binding in the central region [32]. This distribution is similar to that of the non-B-cells within the pancreatic islet. Figure 4 presents immunological evidence that

GABA-activated Cl^- channels are present in rat A-cells. The gross distribution of the immunoreactivity against the β-subunit of the $GABA_A$-receptor Cl^- channel (light grey; [33, 34]) is largely the same as that of glucagon (black) and in a few cases (indicated by arrows) individual cells can be identified that stain with antibodies for both the channel protein and glucagon [cf. Ref. 6]. It is evident, however, that some cells equipped with $GABA_A$-activated Cl^- channels do not contain glucagon. These cells correspond to somatostatin-producing D-cells [6]. GABA-activated Cl^- currents with properties similar to those observed in the A-cell can indeed be recorded from islet cells with D-cell morphology [6]. This supports the suggestion that activation of $GABA_A$-receptor Cl^- channels may be involved in the regulation of both glucagon and somatostatin secretion [35].

To establish a role for the $GABA_A$-activated Cl^- channels in the A-cell, it is important to demonstrate that variations in the activity of the channels are able to influence glucagon secretion. Application of GABA was found to inhibit arginine-stimulated glucagon secretion, an effect which was reversed by the $GABA_A$-receptor antagonist bicuculline [6]. Moreover, diazepam augmented the inhibitory effect of a low concentration of GABA on glucagon secretion (Östenson C-G, Berggren P-O and Rorsman P, unpublished observations). Activation of $GABA_A$-receptor Cl^-channels also appears to play a role in glucose inhibition of glucagon release, and the inhibitory effect of glucose accordingly decreases after blockage of the $GABA_A$-activated Cl^- channels by bicuculline [6].

A working model for the regulation of glucagon secretion

In Fig. 5 we attempt to incorporate the above observations into a working model for the regulation of glucagon release. Elevation of the glucose concentration leads to stimulation of insulin and GABA release from the B-cells. GABA diffuses to the A-cells, binds to the $GABA_A$-receptor Cl^- channels and increases the Cl^- permeability of the A-cell. This results in hyperpolarization, inhibition of electrical activity, reduction of Ca^{2+} influx, decrease in $[Ca^{2+}]_i$ and eventually suppression of glucagon secretion. Preliminary evidence suggests that GABA may also reduce electrical excitability by a direct inhibitory action on the T-type Ca^{2+} channel (unpublished observations). Similar effects have been observed in neurones and are believed to be secondary to chemical modulation of the Ca^{2+} channel by G-proteins [36]. The A-cell metabolizes glucose at a low rate when compared to the B-cell. Nevertheless, it is possible that glucose metabolism is able to directly modulate glucagon secretion by interfering with the intracellular Ca^{2+}-handling or by regulation of $GABA_A$-receptor Cl^- channels. The latter mechanism is suggested by analogy with nerve cells, where the amplitude of the $GABA_A$-receptor Cl^- current is dependent upon the metabolic state of the neurone [37].

Two predictions can be made from the model summarized in Fig. 5. First, any condition leading to stimulation of insulin secretion will automatically

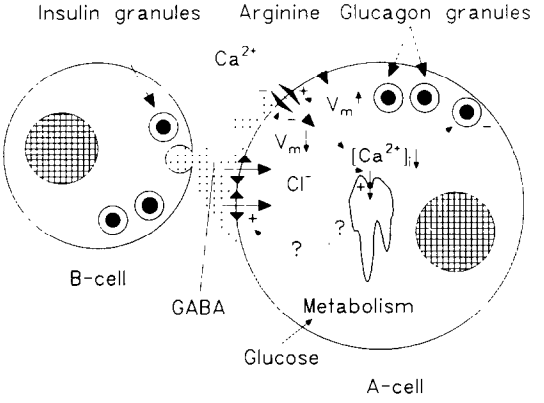

Fig. 5. Model for the regulation of glucagon secretion. The stimulation of insulin secretion leads to the simultaneous release of GABA from the B-cell. GABA diffuses to the A-cell, binds to $GABA_A$ receptors and opens a Cl^- channel. This results in membrane hyperpolarization, inhibition of electrical activity, and the reduction of Ca^{2+} channel activity. Consequently, $[Ca^{2+}]_i$ decreases and glucagon secretion is inhibited. Arginine (positively charged) depolarizes the A-cell by its electrogenic entry. Apart from the indirect modulation of glucagon secretion via GABA release from the B-cell, glucose may also reduce glucagon secretion by: (1) interfering with A-cell $[Ca^{2+}]_i$ handling (e.g. enhanced sequestration into subcellular organelles) or (2) modulating $GABA_A$ receptor Cl^- channels.

reduce glucagon secretion. Second, from the proposed interrelationship between insulin and glucagon release, it follows that glucagon secretion will increase under conditions of impaired B-cell function. This may in itself be sufficient to explain why human diabetes is complicated frequently by hypersecretion of glucagon, which aggravates the hyperglycemia resulting from the lack of insulin [38]. In addition, at low insulin concentrations, gluconeogenetic reactions will be enhanced and lead to increased concentrations of circulating amino acids. These will stimulate glucagon release and thus contribute to hyperglucagonemia. Administration of insulin will inhibit gluconeogenesis, resulting in a reduction of the amino acid concentrations and inhibition of glucagon secretion. The clinical observation that insulin therapy leads to normalization of glucagon release should consequently not be taken as evidence in favor of a direct inhibitory role of insulin on the A-cell. In fact, it has been reported that insulin treatment only prevents glucagon hypersecretion but does not correct the inability of glucose to suppress basal glucagon secretion [39]. This observation may be explained by the model proposed here.

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