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Review

Neurotransmitters and Their Receptors in the Islets of Langerhans of the Pancreas

What Messages Do Acetylcholine, Glutamate, and GABA Transmit?

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Although neurotransmitters are present in pancreatic islets of Langerhans and can be shown to alter hormone secretion, their precise physiological roles in islet function and their cellular mechanisms of action are unclear. Recent research has identified specific neurotransmitter receptor isoforms in islets that may be important physiologically, because selective receptor agonists activate islet ion channels, modify intracellular [Ca^{2+}], and affect secretion. This article focuses on the putative roles of acetylcholine, glutamate, and GABA in islet function. It has been hypothesized that acetylcholine potentiates insulin secretion by either promoting Ca release from cellular stores, activating a store depletion-activated channel, or activating a novel Na channel. GABA and glutamate, in contrast, have been proposed to mediate a novel paracrine signaling pathway whereby α- and β-cells communicate within the islet. The evidence supporting these hypotheses will be critically evaluated.

Key Words: Neurotransmitters; islets of Langerhans; insulin secretion; acetylcholine; GABA; glutamate; NMDA; AMPA; muscarinic receptors.

Introduction

In the past few years, there has been an explosion of interest in the role of neurohormones and their intracellular signaling systems as modulators of glucose-dependent insulin secretion from pancreatic islets of Langerhans *(1–4)*. It thus seemed desirable to review recent findings demonstrating novel actions of several prominent neurotransmitters, particularly those whose importance in the central nervous system, by analogy, are now very well established. Since some excellent reviews of neurohormone-mediated inhibition of insulin secretion have recently appeared or are in press *(3,4)*, this article will not be comprehensive, but will focus on three particular neurotransmitters: acetylcholine (ACh), glutamate and γ-aminobutyric acid (GABA). Since the literature associated with islets and neurotransmitters is vast, we apologize in advance to any of our colleagues whose work we have not cited for brevity's sake.

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Acetylcholine (ACh)

Activation of the parasympathetic branch of the autonomic nervous system has long been known to increase insulin secretion and peripheral glucose uptake *(5)*. ACh acts as a positive modulator of insulin secretion (reviewed in *1*), but like many other modulators, this activity requires glucose metabolism *(6–8)*. ACh action is believed to be mediated by the M3 subtype of the muscarinic receptor. Although most of the evidence implicating this receptor comes from pharmacological studies *(9,10)*, RT-PCR studies of rat islets have identified RNAs encoding both the M3 and M1 muscarinic receptor subtypes, with M3 existing in higher relative abundance *(10)*. Although it has been suggested that nicotinic receptors may have a role in islet function *(11)*, most investigators find that the muscarinic antagonist atropine completely inhibits the effects of ACh in islets. In contrast, the nicotinic agonist nicotine and its antagonist *d*-tubo curarine are usually found to be without effect *(12)*.

ACh has a number of actions on islet β-cells and insulin-secreting islet cell lines, which may individually or in combination potentiate insulin secretion. These cellular mechanisms are shown diagrammatically in Fig. 1. ACh releases Ca from endoplasmic reticulum Ca stores, resulting

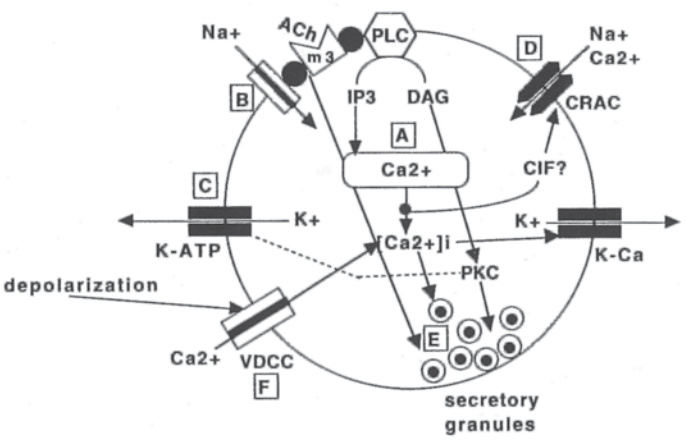

Fig. 1. Possible cholinergic signaling pathways in the pancreatic islet β-cell. Although there is a consensus that ACh initiates a cellular signaling cascade, which starts with the occupation of M3 muscarinic receptors and ends with modulation of the release of insulin from secretory granules, a number of hypotheses have been put forth to account for the intervening steps, including modulation of plasmalemmal ion channels, release of Ca from its intracellular stores, and activation of PKC. Details are provided in the text.

in a rise in $[Ca^{2+}]_i$ *(13–15)*, modulates β-cell ion channels to produce membrane depolarization, enhanced electrical activity, and increased Ca^{2+} influx *(6,8,12)* and ACh may modulate insulin secretion at a distal, Ca-independent site of action *(3)*. These mechanisms will be discussed in turn below.

Release of Ca from Endoplasmic Reticulum Ca Stores

ACh mobilizes intracellular Ca release from endoplasmic reticulum stores in insulin-secreting cells owing to the generation of inositol-1,4,5-tris phosphate (IP_3) following stimulation of phospholipase C (PLC; Fig. 1[A]; *13,14,16–19*). The second metabolite of this pathway, diacylglycerol, also potentiates insulin secretion, suggesting dual control of secretion following receptor activation of PLC *(15,20–22)*. The stimulation of islet phosphoinositide turnover and Ca mobilization by ACh appears to require a pertussus-toxin-insensitive G-protein *(13,14)*.

ACh Depolarizes β-Cells and Modulates Islet Electrical Activity in a Glucose-Dependent Manner

ACh depolarizes islet β-cells, particularly in suprathreshold glucose *(6,8,12)*. Even disparate depolarizing stimuli, such as high external [K], elevated glucose, tolbutamide, or arginine, are able to provide sufficient depolarization to permit maximal ACh action *(8)*. Although it is widely recognized that ACh-induced islet depolarization is an important part of this transmitter's mechanism of action, the mechanism mediating this depolarization is unresolved and has been controversial. It is also interesting to note that some cholinergic agonists have also been reported to increase $[Ca^{2+}]_i$ and/or insulin secretion in certain cell lines without obviously depolarizing the secreting cells *(13,14,23)*.

In mouse islets, the application of ACh or its analogs in basal glucose produces only a small depolarization. However, both the magnitude and the functional effectiveness of ACh-induced depolarization increases with glucose concentration *(6,8,12,24,25)*. In 11.1 m*M* glucose, the application of ACh rapidly depolarizes cells to a plateau voltage level associated with high-frequency spiking. This phase is usually then followed by an increase in plateau frequency, but not plateau fraction. These effects are blocked by the muscarinic antagonist atropine *(6)* and tend to be considerably variable depending on the particular agonist used, its concentration, and the glucose concentration.

Thus, although the application of 10 μ*M* ACh with 11 m*M* glucose was reported to prolong bursting and increase spiking initially *(6,26)*, 1 μ*M* ACh applied with 15 m*M* glucose resulted in a more marked prolongation of the plateau spiking phase *(25)*. In contrast, use of either 10 μ*M* muscarine or 100 μ*M* carbamoylcholine with 11 m*M* glucose produced an initial transient stimulation phase followed by marked hyperpolarization and then a transient cessation of spiking activity. The islets then slowly depolarized and plateau frequency was increased *(26,27)*. Interestingly, the actions of ACh cannot be mimicked by simply raising the concentration of glucose, since elevated glucose increases plateau fraction and decreases plateau frequency, whereas ACh increases frequency without affecting plateau fraction *(6)*.

Although there is wide agreement that ACh increases Ca influx, leading to a rise in $[Ca^{2+}]_i$ and increased net ^{45}Ca uptake *(15,16)*, the mechanism involved is controversial. Clearly this uptake must involve L-type voltage-dependent Ca channels (or VDCCs), since selective L-channel blockers, like nifedipine, nitrendipine, D600, and verapamil, abolish ACh-dependent Ca uptake (*16*; Fig. 1F). However, a number of diverse mechanisms have been hypothesized to account for the ACh-induced cell membrane depolarization, which indirectly opens the VDCCs (Fig. 1,B–D).

First, it has been proposed that ACh, like glucose, depolarizes islets by closing the ATP-sensitive K channels (KATP channels) of the β-cell plasma membrane (Fig. 1C). This was proposed to result from muscarine receptor-mediated activation of PLC and the subsequent activation of PKC by diacylglycerol (DAG; *28*). This hypothesis is supported by the findings that carbachol induces an increase in ^3H-DAG and inositol phospholipid hydrolysis *(20)*, and application of phorbol myristate acetate (PMA; 100 n*M*) or a related DAG analog DC-10 suppresses KATP channel activity and stimulates $[Ca^{2+}]_i$ and insulin secretion in RIN cells *(28)*.

The application of PMA or related products of PLC activation depolarizes RIN m5F cells and triggers

repetitive spiking, although only transiently *(28)*. However, the application of as little as 30 nM PMA was also found to inhibit potassium-stimulated elevation of $[Ca^{2+}]_i$ in this same cell line *(29)*. These inconsistent effects of PMA and related compounds raise doubt that their actions are in fact easily interpretable or are owing to PKC activation.

Furthermore, if the major action of ACh was indeed to close KATP channels through the generation of DAG, then the electrical effects of ACh should qualitatively resemble those of glucose or tolbutamide, both of which are widely accepted KATP channel inhibitors *(30)*. However, neither glucose nor tolbutamide mimics the effects of ACh on islet electrical activity *(6,31)*. Thus, the major actions of ACh on islets are probably mediated by mechanisms other than PKC-induced KATP channel inhibition. PKC is more likely to mediate the distal modulation of insulin secretion associated with cholinergic stimulation (*22,23*; Fig. 1E; *see below*).

Another hypothesis put forward to account for ACh-induced islet depolarization suggests that muscarinic ACh receptors are coupled (perhaps through a G-protein) to a novel Na-permeable ion channel in the β-cell membrane (Fig. 1B); *12*). Activation of this channel following receptor occupation would depolarize islets by mediating an increased net influx of Na^+ *(12)*. Available evidence to date supports the view that Na influx is indeed important for ACh action and can be summarized as follows: the depolarizing action of ACh on islets is reduced or blocked by removing external [Na] *(32)*; ACh increases islet [Na], as evidence by increased ^{22}Na uptake *(12)*, Na content *(33)*, and increased $[Na^+]_i$ *(25,32,34)*. In islets bathed in 3 m*M* glucose, 100 μ*M* ACh slowly elevated intracellular $[Na^+]_i$ from a basal level of 16–30 m*M* within 15 min. The application of Na-free external saline subsequently decreased $[Na^+]_i$ to below its basal concentration *(32)*. In the presence of 15 m*M* glucose, basal $[Na^+]_i$ was reduced (5–10 m*M*), and the subsequent increase associated with ACh was also reduced. This glucose sensitivity presumably reflects increased Na pumping owing to increased glucose metabolism *(34)*. Ignoring Na pumping or buffering, and assuming β-cell diameter to be 12 μm, the elevation in $[Na^+]_i$ observed following ACh in theory must result from the activation ca. 1.35 pA of Na current for 15 min.

Na influx through a Na channel thus appears to be important for ACh-induced islet depolarization, and the activation of these Na channels would then mediate islet depolarization to the voltage threshold of islet voltage-dependent Ca channels. What is the nature of this channel? Although tetrodoxin (TTX)-sensitive Na channels have been described in the mouse *(35–38)*, dog *(39)*, and human β-cells *(39)*, it is unlikely that these Na channels play a role in the action of ACh (but *see 40*), since TTX does not block ACh-induced Na uptake into mouse islets or ACh-induced islet electrical activity *(32)*.

Henquin and colleagues *(8,12,32,34)* have proposed instead that M3 muscarinic receptors are coupled to a novel Na channel. This novel channel might be directly gated by the receptor or might be coupled through a G-protein. Unfortunately, this channel has never been characterized in β-cells or cell lines, although Ashcroft and Rorsman mentioned observing such a channel in passing in their 1989 review *(41)*. The elusiveness of the theorized channel has been suggested to reflect its loss from β-cells when they are cultured *(41)*. If this is true, however, then one would expect a parallel loss of characteristic ACh responses in cultured cells. An alternative is that the channel's unitary conductance is below the level of resolution of currently available patch-clamp amplifiers. At the present time, therefore, the primary weakness of this hypothesis is that the channel has not been characterized.

Evidence Supporting a Ca-Release-Activated Ion Channel in Islets

Another mechanism that has received a good deal of attention of late and has the interesting property that it can link ionic events at the cell surface with biochemical signals within the cell is the hypothesis of a Ca-release-activated Ca or Na current in islet cells. In this mechanism, the release of Ca from intracellular Ca stores owing to IP_3 is coupled to the activation of a calcium release activated current (CRAC) current (*see* Fig. 1D for "CRAC"; *42*) at the surface membrane. It has been suggested from work in other cell types that a Ca influx factor or "CIF" may be generated by store depletion, which could then diffuse to the plasma membrane and activate CRAC channels (*43*; reviewed in *44*). Increased Ca influx mediated by the CRAC channel directly, or owing to VDCC activation following CRAC-mediated cell depolarization would then heuristically serve to refill the recently emptied Ca stores. This mechanism has been called the "capacitance model" of Ca entry by Putney *(45)*.

Evidence for a CRAC-like mechanism in islets comes from observations by Worley et al. *(46)*, who found that the SERCA ATPase inhibitor thapsigargin (Tg) or the application of external solutions containing low Ca activated a nonselective ion current (principally carried by Na). Unfortunately, it is not known whether the putative islet CRAC channels are also activated by ACh, since this was not tested. However, numerous studies show that Tg and related compounds elevate $[Ca^{2+}]_i$ in islets *(18,25,34,46–48)*. Related to these findings is the recent report that the gene encoding the protein "Trp," a potential CRAC channel protein, has been cloned from islets by Qian et al. *(49)*. However, as pointed out by Berridge *(44)* and by Clapham *(50)*, there are reasons to think that Trp may not mediate CRAC, since Trp and CRAC have significantly different channel properties.

The feasibility of a CRAC-type mechanism has been confirmed in theoretical studies as well as in experimental studies of whole-islet electrical activity *(24,47)*. Thus, Bordin et al. *(24)* found that the muscarinic agonist oxo-m stimulated insulin release in 11.2 m*M* glucose, depolarized islets, and increased their burst frequency at 0.1 μ*M*. At 10 μ*M* there was an initial plateau depolarization, followed by a hyperpolarization and then high-frequency bursting. These effects closely resembled those of ACh. Interestingly, when 50 μ*M* oxo-m were applied to islets along with 50 n*M* charybdotoxin (ChTX), the hyperpolarization phase was abolished and was replaced with continuous spiking *(24)*. ChTx is a relatively selective blocker of the large conductance or "BK"-type Ca-activated K channel (KCa) known to be present in islets *(51)*. This finding thus suggests that BK channels may play a role in ACh modulation of islet bursting, even though their importance in general in glucose-dependent bursting has been devaluated (*see 52,53*).

A recent theoretical model of ACh-modulated bursting *(47)* provides further insight into how KCa channels might participate in mediating some of the actions of ACh through CRAC activation *(47)*. These authors found that $[Ca^{2+}]_i$ could rise to higher levels in response to muscarinic agonists, compared to the levels of $[Ca^{2+}]_i$ seen with glucose alone. In the absence of muscarinic stimulation, glucose-induced bursting is produced in this simulation by the cyclic activation and then slow inactivation of the VDCCs *(6,54)*. However, muscarinic stimulation alters this steady-state bursting pattern in this model in several ways. First, store depletion causes a rapid increase in $[Ca^{2+}]_i$, which is without any superimposed oscillations. This initial phase of $[Ca^{2+}]_i$ elevation is sufficient to activate a large number of KCa channels, resulting in an initial silent hyperpolarization phase that lasts tens of seconds. As the endoplasmic reticulum stores continue to deplete, $[Ca^{2+}]_i$ starts to decline slowly owing to Ca efflux, and the membrane begins to depolarize slowly from the initial hyperpolarized potential as CRAC activates. Depolarization proceeds to the point where an initial rapid spiking phase commences. Fast small amplitude $[Ca^{2+}]_i$ oscillations at this time occur owing to Ca spike activation. The growing CRAC current in the model thus provides a depolarizing background current to mediate the fast bursting phase. During this fast bursting phase, KCa channels periodically activate and deactivate, increasing the frequency of the bursts, which repolarize as KCa tracks the fast oscillations in $[Ca^{2+}]_i$. In fact, most of the electrical effects of ACh in the Bertram model are the results of the activation of KCa channels rather than CRAC activation *per se*. The patterns seen with this model could also work with an ACh-activated inward current channel as well, which need not be a CRAC, as long as the channel can contribute net depolarizing drive to the system (A. Sherman, personal communication). Thus, this impressive model can generally account for the increased plateau frequency seen with muscarinic agonists following a period of increased spiking in islets *(47)*.

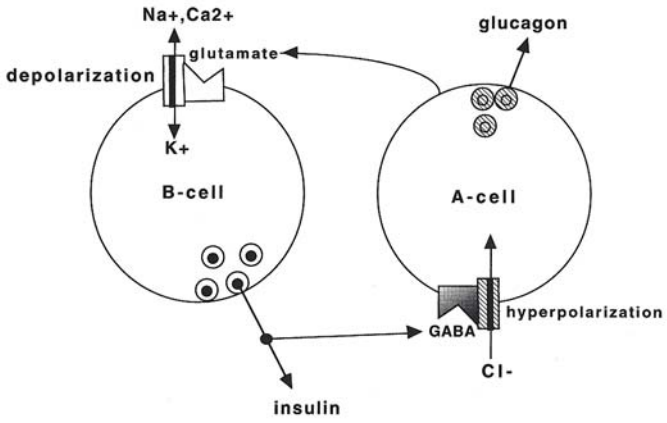

Fig. 2. Hypothetical crosstalk between insulin-containing β-cells and glucagon-containing α-cells mediated by glutamate and GABA. In this model, glutamate released from α-cells depolarizes receptive β-cells by increasing their Na, K, and possibly Ca conductance, whereas GABA coreleased with insulin from β-cells is believed to hyperpolarize α-cells, thus decreasing their action potential frequency, which in turn reduces Ca influx and glucagon secretion. More details about this scheme are provided in the text.

However, there are some issues concerning the model and the experimental evidence supporting it provided in Bertam et al. *(47)*. First, as noted earlier in this article, it is usually the case that muscarinic agonists initially cause rapid spiking following abrupt islet depolarization rather than causing an initial hyperpolarization followed by slow depolarization as predicted by the Bertram model *(6,8,12, 24,26,27)*. However, the model can show this pattern as well with modest parameter variation. Second, one might expect that if Ca release triggers sustained Ca influx into the islet through store depletion and CRAC activation, Tg should closely mimic the electrical effects of ACh, since it also empties the Ca stores. Examination of the experimental data shown in Fig. 2 of Bertram et al. *(47)* shows that consecutive applications of Tg (at 1, 3, and 5 μ*M*) to a bursting islet produced a slow irreversible depolarization, increased plateau frequency and fraction, and only eventually was a continuous spiking from the plateau level obtained. Although depolarization and increased plateau frequency are classic hallmarks of ACh action in islets, increased plateau fraction is not *(6)*, and the slower time-course of these effects did not closely resemble the patterns observed with ACh or muscarinic agonists. The latter instead have an initial enhanced spiking phase, a transient slowing or hyperpolarized phase, followed by depolarization to a period of high frequency or "muscarinic" bursting *(24,26,47)*. Problems with this experiment may reflect the slower diffusion of Tg into islets compared to ACh, which abrogates close comparison between the kinetics of the two agonists (Sherman, personal communication).

Miura et al. *(34)* directly examined the CRAC hypothesis by testing whether ACh raised $[Na^+]_i$ in islets once their intracellular Ca pools were depleted by Tg or cyclopiazonic acid (CPA), another SERCA inhibitor. Although Tg elevated islet $[Ca^{2+}]_i$ in Ca-free solutions, and in separate experiments ACh stimulated a slow rise in islet $[Na^+]_i$, 1 μ*M* Tg had little or no effect on islet $[Na^+]_i$, and pretreatment of islets with Tg had no effect on the changes in $[Na^+]_i$ produced by the subsequent addition of ACh. Interestingly, some elevation of $[Na^+]_i$ following CPA addition was observed, although as for Tg, this did not affect the subsequent response to ACh. These authors used FURA-2 to measure islet $[Ca^{2+}]_i$, and found that 1 μ*M* ACh retained its ability to elicit a sustained elevation in $[Ca^{2+}]_i$ or prolonged$[Ca^{2+}]_i$ spikes even after Tg or CPA was applied to empty the intracellular Ca storage pools. This is not easily compatible with the CRAC hypothesis, since prior emptying of the stored Ca pools should eliminate this signaling component. However, it is not clear that this rules out all possible scenarios involving CRAC and ACh. Thus, Ca pool emptying might be necessary, but not sufficient to trigger a Ca influx component, with another step being necessary. In support of this, Bode and Goke *(48)* found that Tg only evoked both transient (Ca release) and sustained (Ca influx) phases of $[Ca^{2+}]_i$ in RINm5F cells if TPA was first applied to ensure that PKC was activated. Thus, other factors may be permissive in order to produce robust Ca influx following Ca release.

ACh May Trigger Ca Release and Activate KCa Channels in Islet Cells

Another novel mechanism was proposed to account for ACh action in islets by Ämmälä et al. *(55)*. These authors reported that exposure of single mouse β-cells to intracellular GTP or carbamoylcholine triggered periodic activation of transient Ca-activated K currents. These currents were coincident with intracellular $[Ca^{2+}]_i$ transients measured in these cells using FURA-2, even when cells were bathed with Ca-free media, and the currents mediated hyperpolarization of the B-cell membrane potential and slow-voltage oscillations. Both conventional BK-type KCa channels as well as smaller TEA- and ChTx-insensitive K channels of a novel type were found to be activated *(55)*. In addition to being activated by external cholinergic receptor occupation, intracellular IP_3 could evoke this current through periodic intracellular Ca release. The authors suggested that this novel mechanism could account for islet bursting being periodic, as well as for muscarinic stimulation of islet function.

However, as pointed out by Cook *(56)*, the actions of carbamylcholine reported by Ämmälä et al. *(55)* did not resemble the well-known classic action of ACh on intact islets, which is depolarizing and stimulatory *(56)*. In addition, the pacemaker mechanism of Ämmälä et al. *(55)* is essentially voltage-independent, whereas the islet pacemaker mechanism is voltage-dependent *(52,57)*.

It is not yet known whether these oscillations are a consistent feature of single-islet β-cells. In addition, there is now evidence to suggest that distinguishing electrical (i.e., ionic) oscillations at the plasma membrane vs biochemical oscillations within the cell might not be simple, since membrane potential appears to modify the phosphoinositide levels of insulin-secreting βTC-3 cells *(19,58)*.

ACh Inhibits Islet Cell Ca Channels

A recent study demonstrated that ACh was also able to inhibit the Ca currents of mouse β-cells *(59)*. These authors measured Ca current using whole-cell techniques and found that 0.25–250 μ*M* ACh inhibited peak Ca current. This inhibition involved a G-protein, since GTP-γ-S in the pipet shifted the dose–response to ACh to the left and produced irreversible inhibition of the Ca current *(60)*. In contrast, GDP-β-S blocked the action of ACh, which was expected since this analog blocks G-protein activation. The G-protein involved was not pertussis toxin- or cholera toxin-sensitive.

The physiological relevance of ACh-induced Ca current inhibition is somewhat puzzling, since ACh as we have seen has been mainly found to be a potentiator of insulin secretion, as discussed by Gilon et al. *(59)*. Although there are conditions in which an inhibitory component of ACh's action can be unmasked, especially at high doses *(25)*, the significance of this action is also unclear, although it may be protective for a cell in order to prevent undue Ca loading following stimulation with ACh. Electrical measurements show that at 100 μ*M* ACh, islets become very depolarized and Ca spike amplitude is reduced *(25)*. Whether this is a direct effect of Ca channel inhibition or results from excessive depolarization or increased electrical shunting is uncertain.

ACh Modulates Insulin Secretion at a Distal Site of Action

Recent studies also suggest that ACh can stimulate insulin release from RIN m5F cells at a "distal" or Ca-independent site of action *(23)*. These authors thus could identify an action of carbachol that was apparently dissociated from actions mediated by $[Ca^{2+}]_i$ or PKC activity (Fig. 1E). $[Ca^{2+}]_i$ was measured in a cuvet filled with RIN cells using FURA-2, and membrane potential was monitored with the dye bisoxonol. Under these conditions, carbachol transiently increased $[Ca^{2+}]_i$ as did TPA. However, nitrendipine did not block the effects of carbachol, but blocked those of tumor-promoting agent (TPA). The effects of carbachol (but not TPA) also persisted after external [Ca] was lowered with [Ethylene-bis(oxyethylenenitrilo)]tetraacetic acid (EGTA). Although TPA application depolarized the RIN cells, carbachol failed to do so *(23)*.

Tg raised $[Ca^{2+}]_i$ and totally blocked the action of carbachol to elevate $[Ca^{2+}]_i$, as did the calmodulin inhibitor W7. However, the major phase of insulin release stimulated by carbachol was largely unaffected by altering Ca, adding W7, or downregulating PKC activity. Thus, although adding Tg to empty Ca stores interfered with the ability of carbachol to raise $[Ca^{2+}]_i$, carbachol stimulation of insulin secretion was unaffected by these conditions. Thus, the authors argued that the effect of carbachol of increasing Ca was thus uncoupled from carbachol stimulation of insulin secretion, and that the agonist must act at a step distal to $[Ca^{2+}]_i$ or PKC signaling steps. However, it was surprising that carbachol failed to stimulate Ca influx or depolarize these particular cells. This suggests that this novel mechanism may be peculiar to this particular cell line and not directly comparable to normal mouse or rat islets.

Although IP_3 clearly releases Ca from endoplasmic reticulum stores in islet cells to trigger at least a transient phase of secretion, there is ample evidence that the other branch of the pathway, phospholipase C activation, is also of importance. Thus, analogs of diacylglycerol have been shown to trigger or modulate $[Ca^{2+}]_i$ oscillations *(60,61)*, sensitize the insulin secretory mechanism to elevations in $[Ca^{2+}]_i$ *(15,22,55)*, depolarize β-cells *(23,28)*, possibly via closure of KATP channels *(28)*, inhibit Ca channels *(29)*, and modulate capacitative Ca current entry *(48)*. A number of studies have examined protein kinase C in β-cells, including its various isoforms (*see 62*). Thus, as with IP_3 formation and action on $[Ca^{2+}]_i$, there is formidable literature documenting the actions of agents thought to activate PKC activity in islet cells.

Islet β-Cells and Insulin-Secreting Cell Lines Express Ionotropic Glutamate Receptors

Glutamate receptors have been subdivided into two major classes in neurons, ionotropic and metabotropic, the former being receptor channels that directly mediate ionic fluxes, and the latter consisting of receptors tied to different intracellular signaling pathways *(63)*. Ionotropic receptors can be further divided by their sensitivity to selective agonists into two subclasses: the NMDA type and the non-NMDA type *(63–65)*. The latter consist of AMPA and kainate receptors. These different glutamate receptors are all depolarizing or excitatory in neurons, but subserve somewhat different functions in neuronal signaling. The cloning of many of the individual subunits of these receptors has produced an impressive body of knowledge concerning the localization and possible role of these proteins in synaptic functioning in the mammalian brain *(63)*.

Of the non-NMDA-type receptors, the AMPA subclass consists of the GluR 1-4 (or GluRA-D) isoforms *(63–66)*, but the kainate receptor isoforms are termed GluR 5-7, KA1, and KA-2. KA-1,2 do not form functional receptors unless they are coexpressed with GluR5 or 6. For NMDA receptors, functional channels consist of an NMDAR1 subunit plus one of the NMDAR2A-D isoforms. Each of the major types of glutamate receptor channels is believed to be a heteromultimer of different subunits *(65)*.

Using RT-PCR, electrophysiology, $[Ca^{2+}]_i$ measurements, and an insulin secretion assay, Gonoi et al. *(67)* obtained evidence for the presence of functional ionotropic glutamate receptors in insulin-secreting MIN6 cells *(67)*. Application of the agonists AMPA, kainate, or NMDA depolarized MIN6 cells by 5–15 mV, and using voltage-clamp, triggered 20–90 pA inward currents. As expected for the NMDA receptor, application of external Mg^{2+} or Zn^{2+} inhibited this current, as did the NMDA-selective antagonists AP-5, 7-Cl-KYNA, and MK-801 *(67)*. Kainate and AMPA currents, on the other hand, were blocked by the non-NMDA antagonist CNQX. Corresponding increases in $[Ca^{2+}]_i$ produced by these agonists were likely owing to Ca influx into the cell, since they were blocked by lowering external [Ca]. Somewhat surprisingly, however, AMPA currents and AMPA-induced rises in $[Ca^{2+}]_i$ did not exhibit the classic desensitization expected for AMPA receptors (reviewed in *64*). It is possible that the AMPA receptors studied were already in a partially desensitized state owing to insufficiently fast drug application. This would lead to an underestimate of the number of AMPA receptors in the membrane.

In terms of function, Gonoi et al. *(67)* found that 3.3 m*M* glucose, the application of 500 μ*M* kainate, AMPA, or NMDA also potentiated insulin secretion, in the rank order of kainate > AMPA > NMDA (–Mg) > NMDA (+Mg). As for the specific receptor molecules involved, RT-PCR showed that MIN6 cells were positive for the NMDA, GluR2 (or GluB), GluR3 (or GluC), KA-2, NMDAR1, NMDAR2C, and NMDAR2D receptor subunits although NMDAR2C was present at very low levels. This work thus showed for the first time that nonneuronal MIN6 cells had endogenous neuron-like ionotropic glutamate receptor channels, which could play a functional role in the control of insulin secretion.

Molnár et al. *(68)* used immunoblotting techniques, with antibodies specific for particular glutamate receptor subunits, and measured binding of cell fragments to tritiated AMPA or kainate to test for the presence and possible function of glutamate receptor channels in MIN6, HIT T-15, RINm5f cells, and rat islets. Their goal was to determine whether the proteins encoded for by glutamate receptors were expressed in these cells.

Molnár et al. *(68)* detected glycosylated forms of NMDAR1 and KA-2 in all preparations tested, but did not detect GluR1. Binding studies revealed some selective binding of glutamate agonists to MIN and RIN cells, although the amount of binding observed was much lower than in brain tissue.

The authors next examined whether glutamate receptor activation affected insulin secretion from rat islets. In

3.3 m*M* glucose, 0.5-m*M* doses of glutamate, NMDA, or kainate were found to have no effect on insulin secretion, whereas application of 0.5 m*M* NMDA (but not the kainate or glutamate) in 8.3 m*M* glucose caused a 47% increase in secretion. In 16.7 m*M* glucose, NMDA caused a 37% increase in secretion, whereas kainate had a small, but insignificant potentiating action and glutamate was totally without effect *(68)*. Given these modest actions of glutamate and the relatively low levels of receptor expression found, the authors cautioned that the functional importance of glutamate receptors for islet function was uncertain.

Inagaki et al. *(69)* also examined glutamate receptor levels in rat pancreatic islets and RINm5f cells using RT-PCR. They assessed possible functional implications using $[Ca^{2+}]_i$ measurements, cell electrophysiology, and an assay of insulin secretion. They found high levels of GluR2 and 3 expression in islets, but only low levels of GluR-1, in confirmation of the findings of Molnár et al. *(68)*. However, Inagaki et al. *(69)* also obtained evidence suggesting that GluR6, GluR7, KA-2, NMDAR1, 2A, 2C, and 2D were all expressed in rat islets. In contrast, NMDAR2A and 2C were only present at very low levels. RIN cell expression was different, since these cells from rat islets expressed GluR2, GluR5, KA-1, NMDAR1, and NMDAR2D. This is interesting, since RIN cells were initially derived from rat islets.

Exposure of rat islet cells to either kainate, AMPA, or NMDA raised $[Ca^{2+}]_i$ and depolarized the cell membrane potential. None of the responses to AMPA showed rapid desensitization, a characteristic of this channel *(69)*. Application of 0.01–1 m*M* kainate or AMPA in 8.3 m*M* glucose potentiated secretion, but NMDA did not. These authors also sublocalized putative glutamate-releasing cells in the rat islets using antiglutaminase immunoreactivity, and found positive staining in the islet periphery and colocalization of glutaminase and glucagon. These findings suggest glutamate synthesis occurs in glucagon-positive α-cells as well as neurons of the intrapancreatic ganglia *(69)*. The authors suggested that glutamate release from α-cells or neurons stimulates insulin secretion via the activation of glutamate receptors on the β-cell, thus participating in the paracrine regulation of insulin secretion (Fig. 2).

Weaver et al. *(66)* examined the expression of different glutamate receptor subunits in rat islets using immunocytohistochemistry in conjunction with laser scanning confocal microscopy, Western blot analysis, and electrophysiology. They also tested whether glutamate receptor subunits were colocalized with each of the four islet hormones (insulin, glucagon, somatostatin, and pancreatic polypeptide), an added strength of their approach.

Antibody specific for GluR2/3 (RB/C in their nomenclature) strongly stained large areas in the center of the islet and the mantle, but acinar tissue was spared. GluR2/3 staining in the islet core was intense and appeared to be localized to α-, β-, and PP but not δ-cells. However, GluR1 staining was found to be either completely absent or very weak, in agreement with the other studies cited earlier. Signals for the kainate receptor subunits GluR6 and R7 were observed in α-, but not β- or δ-cells *(66)*. Immunoreactivity against KA2 was only obtained in the δ-cells.

Positive staining corresponded to the AMPA receptor subunit GluR4, and the NMDA subunit NMDAR1 was absent. This suggests that functional AMPA receptors in rat islets consist of GluR2 and 3 with possibly some GluR1, whereas functional NMDA receptors are lacking in these cells. GluR2/3, 6/7, and KA2 immunoreactivity was also detected via Western blotting using islet membrane homogenates. Interestingly, GluR 6/7 or KA-2 could not be detected with this approach when whole isolated islets were used, possibly owing to the limited amount of tissue.

The application of 300 μ*M* L-glutamate to a subpopulation (27%) of single rat islet cells transiently depolarized the cells, sometimes triggering Ca action potentials *(66)*. Exposing cells to glutamate, AMPA, or kainate under voltage-clamp conditions produced very small inward currents (e.g., ≈2 pA). However, cyclothiazide, which blocks the desensitization of neuronal AMPA channels, greatly potentiated these AMPA currents, which increased in amplitude to ≈25 pA *(67)*. Islet glutamate receptors could be activated by AMPA, kainate, or L-glutamate and were blocked by CNQX. The current mediated by the channels reversed at –2.5 mV, which would account for the depolarizing effect of glutamate and AMPA agonists in islets, and the channels showed variable degrees of rectification. These results thus demonstrate that AMPA and kainate receptors in islets are functional and resemble the glutamate receptors of neurons.

Despite this strong evidence for glutamate receptors in islets, the significance of the receptors for islet physiology remains unresolved. Under physiological conditions, the responses to glutamate observed were very small and transient *(66)*. Although one cannot rule out that small currents can be important in electrically tight cells, such as islet cells *(52,70)*, it seems unlikely that currents that desensitize within 1 s would be an effective and appropriate signaling system in islets, which can exhibit glucose-dependent slow-plateau depolarizations lasting tens of seconds *(6,41)*. This means that any depolarizing effect of glutamate would be small and quickly damped. However, if sustained inward current components are activated by L-glutamate, as suggested by the αTC cell data of Weaver et al. *(66)*, these might be capable of modifying electrical activity, perhaps to bring cells to their firing threshold, as was sometimes observed by these authors.

Bertrand et al. *(71)* had earlier carried out an extensive functional study of the role of glutamate receptors in islet function. These authors tested whether glutamate receptor agonists modulate insulin secretion from isolated perfused rat pancreas *(71)*. They found that 50–4000 μ*M* L-glutamate applied in 8.3 m*M* glucose provoked a transient (2-min)

phase of mild insulin secretion, whereas glutamate was ineffective when applied with 2.8 m*M* glucose. This suggested that glutamate was a potentiator, but not an initiator of secretion. Kainate, AMPA, or quisqualate all increased secretion, but NMDA had no effect. The effects of kainate and L-glutamate were blocked by CNQX, whereas the NMDA antagonist MK-801 had no effect. To rule out that these effects were mediated by the nervous system, atropine and tetrodotoxin were added to block neuronal muscarine receptors and spike discharges, respectively. Neither treatment interfered with the action of the glutamate agonists. Therefore, the authors concluded that islet AMPA receptors are physiological potentiators of insulin secretion *(71)*.

This group subsequently showed that AMPA receptors were also capable of modulating glucagon secretion from the perfused pancreas, although in this case, glutamate was found to be a much weaker secretagog for glucagon compared to insulin. In addition, although insulin secretion was more strongly affected when applied with 8.3 m*M* glucose, stimulation of glucagon secretion was more pronounced with 2.8 m*M* glucose *(72)*. Recently, this group revisited the role of AMPA receptors in insulin secretion by looking at secretion in vivo. Intravenous or orally administered glutamate was observed to stimulate insulin secretion and improve glucose tolerance *(73)*. It is difficult to know whether glutamate's site of action in this study occurred at the level of the islets, and was thus direct, or whether these effects in some way were mediated indirectly by the nervous system.

Johnson et al. *(74)* also examined the possible modulation of insulin secretion by glutamate using perifused rat islets. They reported an abstract form that at doses <1 m*M* L-glutamate lacked a significant effect on secretion regardless of whether 3.3 or 8.3 m*M* glucose was used. However, at doses of 1 m*M* or higher, L-glutamate produced a transient and brisk increase in insulin secretion, which was dose-dependent. In contrast, glutamate did not affect basal insulin secretion. This report thus strengthens the possibility that glutamate receptors may be of importance for insulin secretion.

In summary, there is ample evidence from physiological as well as molecular studies that glutamate receptors, particularly those of the AMPA and kainate subtype, are present in islet cells and may participate in the control of insulin secretion. However, although there is some evidence that α-cells and pancreatic neurons possess the enzyme that synthesizes glutamate *(69)*, there is at present no evidence demonstrating that glutamate is released from these sources under physiological conditions. Although it is possible that dietary glutamate levels may also activate AMPA or other glutamate receptors, this remains to be determined. Indeed, one group has raised serious questions regarding the significance of the glutamate receptors given their low levels of expression in islet cells *(68)*. Thus, at least for the present, the significance of glutamate signaling for islet function remains somewhat unclear, especially when compared to the experimental evidence supporting a functional role for ACh or adrenergic receptors in islet function *(4)*.

GABA Receptors Are Expressed in Islets and Insulin-Secreting Cell Lines

It has been known for some time that plasma glucose concentration is inversely related to glucagon secretion, with low plasma glucose being stimulatory and high glucose being inhibitory for glucagon secretion. Glucagon secreted from islet α-cells *(2)* produces an elevation in plasma glucose, primarily via its actions on the liver. In addition, it has long been known that islet tissue contains large amounts of the neurotransmitter GABA and its synthetic enzyme glutamic acid decarboxylase (GAD), although the physiological function of these molecules in islets is not known (reviewed in *75*). However, a possible role for GABA as a regulator of pancreatic hormone release was proposed by several early workers (e.g., *76,77*).

More recently, Rorsman et al. *(2,78)* proposed that the restraining action of glucose on glucagon secretion from α-cells may result from the activation of $GABA_A$ channels present in guinea pig islet α-cells *(78)*. In the brain, $GABA_A$ receptor channels are heteromultimeric and mediate inhibitory neurotransmission *(79,80)*. The release of the GABA from presynaptic neurons inhibits the firing of postsynaptic neurons by increasing their chloride conductance and hyperpolarizing the postsynaptic neurons.

Although islet cells do not form chemical synapses with each other, Rorsman et al. *(78)* hypothesized that an analogous mechanism could exist in the islet if glucagon-containing α-cells expressed the inhibitory $GABA_A$ receptor channel, but glucose-activated β-cells synthesized and coreleased GABA with insulin. The GABA thus released with insulin following a rise in plasma glucose would in turn inhibit α-cell glucagon secretion owing to GABA channel activation, cell hyperpolarized, and decreased firing of Ca-dependent action potentials. These α-cells could either be in the vicinity of the β-cells, which released the GABA, or receive some neuronal GABAergic input *(78)*.

A number of lines of evidence support this intriguing hypothesis. First, immunochemistry revealed positive staining for the protein corresponding to the α2-subunit of the GABA channel was found to be expressed in α-cells and somatostatin-containing δ-cells, but not in β-cells. More recently, Yang et al. *(81)*, using RT-PCR, found that isoforms of the α2, β3, and γ1-subunits of the GABA channel were present in human pancreatic tissue. Second, Rorsman et al. *(78)* found that exogenous application of 100 μ*M* GABA to single α-cells hyperpolarized them by ≈15 mV and decreased their action potential frequency. Under voltage-clamp, GABA application activated a

desensitizing whole-cell current of variable amplitude (which ranged from few to several hundreds of pAs). This amount reversed near the expected Cl Nernst potential, was increased by the $GABA_A$ channel potentiator diazepam, and was blocked by the $GABA_A$ antagonists picrotoxin or bicuculine *(78)*. Using single-channel methods, a single channel having a unit conductance of 18–29 pS was observed, which resembled the $GABA_A$ channel conductance measured in nervous tissue. However, it was not clear that the islet GABA channel displayed the multiple conductance states, which are also characteristic *(82,83)*. These characteristics taken together suggest that a neuronal GABA channel exists in guinea pig α-cells isolated from guinea pig islets.

Although these authors could not demonstrate that glucose stimulates GABA release, they found some modest effects of exogenous GABA or bicuculine on glucagon secretion. Thus, following the stimulation of glucagon secretion by 10 m*M* arginine in the presence of 1.7 m*M* glucose, the application of 100 μ*M* GABA caused a 27% decrease in glucagon secretion, and part of this inhibition was blunted by bicuculine. Although it was stated that this represented a 70% increase in arginine-stimulated secretion, it was not clear that whether GABA may have also altered glucagon secretion in 1.7 glucose. Interestingly, glucagon secretion was also inhibited when glucose was increased to 16.7 m*M* without arginine being present; this inhibition was itself partly, but not fully relieved by bicuculine. This suggests that $GABA_A$ channels are involved in glucose suppression of glucagon release *(78)*.

This issue was re-examined by Gilon et al. *(75)* in some detail using different approaches and preparations. These authors initially used immunocytohistochemistry to confirm that the islet contained GABA and GAD. GAD staining appeared to be selectively localized to the core, but not mantle of the islet, whereas interestingly, GABA stain was more uniformly distributed; neither GABA nor GAD was present in exocrine tissue.

The inhibitory effects of GABA or muscimol on glucagon secretion reported by these authors were quite modest (≈20%), using isolated mouse islets. Moreover, this small degree of inhibition was not blocked by bicuculine, even when doses up to 100 μ*M* were used. This is in direct contradiction to the findings of Rorsman et al. *(70)*. $GABA_A$ agonists also had no effect on glucose-stimulated insulin or somatostatin secretion, or on islet electrical activity.

Using isolated rat islets, Gilon et al. *(75)* found that for the mouse, neither GABA nor muscimol altered insulin or somatostatin secretion. They could not test the hypothesis that GABA mediated glucose-dependent inhibition of glucagon release, since glucose did not inhibit glucagon secretion in this preparation. They thus studied perfused rat pancreas instead, and found that although muscimol indeed suppressed the biphasic release of glucagon following arginine stimulation, the effect was irreversible. Perfusion of the pancreas with 2.8 m*M* glucose and arginine produced a decrease in glucagon secretion, which was again irreversibly inhibited by muscimol and only slightly decreased by bicuculine. The very robust suppression of glucagon secretion associated with elevation of glucose from 2.8 to 16.7 m*M* glucose was unaffected by bicuculine, suggesting that activation of $GABA_A$ receptors by GABA release cannot be mediating glucose suppression of glucagon release. Although it is not clear why such disparate results have been obtained by these two laboratories, it is possible that the mechanisms regulating glucagon release from guinea pig islets are different from those of mouse or rat, although this remains to be determined.

More recently, Gaskins et al. *(84)* produced some interesting findings concerning the GABA hypothesis. First, these authors succeeded in demonstrating that the insulin-secreting β-cell line βTC6 can release GABA in response to elevated glucose. Incubation of 70% confluent βTC6 cells for 12 h in medium containing 10 m*M* glucose followed by exposure to 10 m*M* glucose for 15–120 min released ≈100 n*M* GABA, as assayed via HPLC. Challenging cells with 1 m*M* glucose in the later period released less GABA compared to when 10 m*M* glucose was used. If the cells were incubated for 12 h in 1 m*M* glucose, they released less GABA when challenged later with 10 m*M* glucose, suggesting that glucose is required for long-term GABA synthesis *(84)*.

These authors next exposed the glucagon-secreting αTC6 cells to concentrations of GABA corresponding to those released from the βTC6 cultures by glucose. In support of the GABA hypothesis, 50–200 n*M* GABA suppressed glucagon release, with near 50% inhibition produced by 100 n*M* GABA. This suppression was reversed by bicuculine, as found by Rorsman et al. *(70)*. Glucagon secretion was also suppressed by increasing glucose from 1 to 10 m*M*; however, this suppression was not affected by bicuculine, suggesting that GABA channels do not mediate glucose-mediated inhibition of glucagon secretion *(84)*. This is consistent with the findings of Gilon et al. *(75)*, who again found that bicuculine did not interfere with glucose suppression of glucagon secretion from perfused rat pancreas. One concern, however, is that the GABA concentrations, which were released from βTC6 cells and which inhibited glucagon secretion from αTC6 cells, were 1000 times higher than the concentrations of GABA found to elicit Cl^- ion currents in guinea pig islet cells *(78)*.

Using single-cell microspectrofluorimetry to assay $[Ca^{2+}]_i$, Berts et al. *(85)* reported that as many as 60% of single glucagon-containing α-cells from mouse islets displayed spontaneous Ca^{2+} oscillations having frequencies ranging from 0.1 to 0 min^{-1} in 3 m*M* glucose. These oscillations were blocked by the Ca channel blocker methoxyverapamil or by 20 m*M* glucose plus 2–200 ng/mL insulin, but they were unaffected by 0.1–1 m*M* GABA, which refutes the hypothesis that GABA released with insulin

from β-cells suppresses glucagon secretion from α-cells. Although neither glucagon release nor Ca spiking was measured in this study, one would expect that exogenous GABA should lower $[Ca^{2+}]_i$ in α-cells if GABA is involved in suppressing glucagon release from these cells.

Summary and Conclusions

We have seen that recent research has provided evidence for a number of neurotransmitter receptors and putative signaling pathways in the islets of Langerhans. These mechanisms may come into play as physiological modulators of islet hormone secretion. For ACh, its action is mediated by an M3 muscarinic receptor on the β-cell and involves Ca^{2+} release via IP_3 generation and some mechanism capable of producing sustained Ca^{2+} entry and depolarization. The ionic mechanism producing depolarization, however, is controversial as is the relative importance of Ca^{2+} release and influx for ACh action on insulin secretion.

As for glutaminergic modulation, the situation is far less clear. Certainly there is a general consensus that non-NMDA-type ionotropic glutamate receptors are expressed in β-cells and that these are also manifested as functional ion channels, which mainly resemble those of neurons. However, the currents mediated by these channels are small and transient, and there is no evidence that glutamate is released under physiological conditions from islet cells or from neuronal structures within the islet. Following the criteria defined for the identification of a neurohormone *(1)*, it may be premature to identify glutamate as an islet neurohormone.

Although GABA and GAD are present in islets and can be sublocalized to β-cells, suggesting some function for GABA signals in islets, only recently have studies shown that the $GABA_A$ receptor is present in glucagon-containing islet α-cells. Analogously to the glutamate receptors of β-cells, functional GABA-gated Cl channel currents have been observed in islet α-cells exposed to exogenous GABA. However, demonstrating that glucose-stimulated GABA release from β-cells has been more difficult to prove, and it remains controversial whether GABA mediates the glucose-dependent inhibition of glucagon secretion from α-cells under physiological conditions.

It has been proposed that glucose-stimulated β-cells corelease GABA with insulin. This results in the opening of GABA receptor channels on neighboring α-cells, which suppresses glucagon secretion. This occurs because as the α-cells hyperpolarize, the frequency of their Ca-dependent action potentials is reduced *(78)*. In contrast, activation of α-cells prompts the release of glutamate onto β-cells, which produces cell depolarization owing to AMPA and/or kainate channel activation. This added depolarization then increases the frequency of β-cell Ca action potentials and potentiates Ca influx and insulin secretion. Thus, GABA and glutamate would mediate a novel two-way intraislet paracrine signaling system where, as in neurons, the former would act as an inhibitory transmitter and the latter would be an excitatory one.

In summary, many neurotransmitter receptor isoforms and their corresponding agonist-activated ion currents have now been demonstrated in islet cells and insulin-secreting cell lines. Although it has been perhaps surprisingly straightforward to find that the isoforms of many neurotransmitter receptors are present in insulin-secreting cells or to observe agonist-gated ion currents, obtaining definitive proof that these transmitters are important for pancreatic islet function has been difficult to establish. In the absence of additional evidence then, we still cannot rule out the possibility that these molecules might subserve different functional roles in the islets of Langerhans than in the central nervous system. Future progress will undoubtedly rely on increased refinements in our use of molecular techniques, the development of more selective receptor agonists and antagonists, and consistent attention paid to the overall physiology of the pancreatic islet.

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