

Evolving insights regarding mechanisms for the inhibition of insulin release by norepinephrine and heterotrimeric G proteins

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Straub SG, Sharp GW. Evolving insights regarding mechanisms for the inhibition of insulin release by norepinephrine and heterotrimeric G proteins. *Am J Physiol Cell Physiol* 302: C1687–C1698, 2012. First published April 4, 2012; doi:10.1152/ajpcell.00282.2011.—Norepinephrine has for many years been known to have three major effects on the pancreatic β -cell which lead to the inhibition of insulin release. These are activation of K^+ channels to hyperpolarize the cell and prevent the gating of voltage-dependent Ca^{2+} channels that increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and trigger release; inhibition of adenylyl cyclases, thus preventing the augmentation of stimulated insulin release by cyclic AMP; and a “distal” effect that occurs downstream of increased $[Ca^{2+}]_i$ to inhibit exocytosis. All three are mediated by the pertussis toxin (PTX)-sensitive heterotrimeric Gi and Go proteins. The distal inhibitory effect on exocytosis is now known to be due to the binding of G protein $\beta\gamma$ subunits to the synaptosomal-associated protein of 25 kDa (SNAP-25) on the soluble NSF attachment protein receptor (SNARE) complex. Recent studies have uncovered two more actions of norepinephrine on the β -cell: 1) retardation of the refilling of the readily releasable granule pool after it has been discharged, an action that is mediated by $G\alpha_{i1}$ and/or $G\alpha_{i2}$; and 2) inhibition of endocytosis that is mediated by Gz. Of importance also are new findings that $G\alpha_o$ regulates the number of docked granules in the β -cell, and that $G\alpha_{o2}$ maintains a tonic inhibitory influence on secretion. The latter provides another explanation as to why PTX, which blocks the effect of $G\alpha_{o2}$, was initially called “islet activating protein.” Finally, there is clear evidence that overexpression of α_{2A} -adrenergic receptors in β -cells can cause type 2 diabetes.

pancreatic β -cell; K^+ channels; adenylyl cyclases; exocytosis; granule pools; endocytosis

NOREPINEPHRINE IS RESPONSIBLE for multiple effects in the body as it acts as a hormone after its release from the adrenal medulla along with epinephrine, and as a neurotransmitter when released by the central and sympathetic nervous systems. It has important functions on the cardiovascular system, on muscle, liver, and adipose tissue, and in the control of whole body metabolism. This review, however, focuses on the effects of norepinephrine on the pancreatic β -cell and recent advances in our knowledge. Norepinephrine has three major effects on the β -cell that lead to the inhibition of insulin release (65, 74, 102, 104). It activates K^+ channels to hyperpolarize the cell. This prevents or reverses depolarization and the consequent gating of voltage-dependent Ca^{2+} channels that increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and trigger insulin release. It inhibits adenylyl cyclases, thus preventing the augmentation of stimulated insulin release, and it has a “distal” effect that occurs downstream of increased $[Ca^{2+}]_i$ to inhibit exocytosis similar to that of serotonin in the central nervous system (CNS) (7) and endothelin in pituitary lactotrophs (3). There are additional reports that in some clonal β -cell lines the inhibitors reduce the activity of the L-type Ca^{2+} channels (38, 43). However, this effect has not been reported in primary

β -cells. That the α_{2A} -adrenergic receptor is responsible for these effects was deduced originally from studies with selective receptor agonists and antagonists (4, 83) and later from data on knockout mice (44, 89). Interestingly, while there is general agreement that the α_{2A} -adrenergic receptor is the primary mediator of the inhibitory effects of norepinephrine, there is evidence that the α_{2C} -adrenergic receptor could play a small role (89). Whether this is direct or indirect is not known. All the effects of norepinephrine to inhibit insulin release are mediated by activation of pertussis toxin (PTX)-sensitive heterotrimeric Gi and Go proteins (52–54, 61).

The effect of PTX to block hormonal inhibition of insulin release was first demonstrated in mice sensitized to PTX during vaccination studies in the 1960s (33, 118). Since 1978 when PTX was identified as the active principal in pertussis vaccines and its action was defined (55, 56, 133), it has been widely used to define signal transduction pathways that are mediated by the Gi/Go proteins. In the case of norepinephrine, all of its α_2 -adrenergic effects to inhibit insulin release are blocked by the treatment of animals, tissues, or cells with PTX (61). The Gi/Go proteins are ADP-ribosylated by PTX, a modification that renders them unable to interact with their receptors, as for example the α_2 -adrenergic receptors. PTX was initially named “islet activating protein” because of the enhanced glucose-stimulated insulin secretion that followed PTX treatment (52–54). One explanation for this activating effect is tonic inhibi-

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tion by an endogenous islet inhibitor such as ghrelin that acts via $G\alpha_{i2}$ (16) and has its inhibitory effect blocked by PTX. However, another explanation has emerged recently from studies on mice lacking $G\alpha_{o2}$ (127). It was found that these mice, but not mice that were lacking other G proteins such as $G\alpha_{o1}$ or $G\alpha_i$ proteins, had increased insulin output in response to glucose stimulation. Whether this is tonic inhibition by another endogenous islet hormone activating $G\alpha_{o2}$ (another because ghrelin selectively activates $G\alpha_{i2}$) or is due solely to a constitutive inhibitory effect of $G\alpha_{o2}$ remains to be determined.

Recently, two novel effects of norepinephrine have been uncovered. These are 1) retardation of the refilling of the readily releasable granule pool (RRP), which obviously reinforces the distal inhibitory effect (135); and 2) inhibition of endocytosis (136). Of interest is the fact that while all the previously known effects of norepinephrine, and the recently discovered effect on the RRP, are due to activation of the PTX-sensitive Gi/Go proteins, the inhibition of endocytosis is due to the activation of Gz, the only member of the Gi/Go family of G proteins to be unaffected by PTX. This article reviews the effects of norepinephrine on K^+ channels, adenylyl cyclases, exocytosis, the RRP, and endocytosis in the β -cell together with the roles played by individual G proteins.

Norepinephrine Activation of K^+ Channels

Activation of the α_{2A} -adrenergic receptor in the β -cell results in hyperpolarization and increased K^+ efflux. This is due to activation of K^+ channels (90) and primarily to the ATP-sensitive K^+ (K_{ATP}) channel. However, it has also been shown that mouse β -cell electrical activity is suppressed by activation of a sulfonylurea-insensitive low-conductance K^+ channel distinct from the K_{ATP} channel but again by a G protein-dependent mechanism (98). Strong evidence for a role for ion channels other than K_{ATP} channels comes from studies with sulfonylurea receptor-1 (SUR-1) knockout mice with nonfunctional K_{ATP} channels (119). β -Cells from these mice are hyperpolarized by activation of α_2 -adrenergic receptors by epinephrine, again with PTX sensitivity (107). The expression and precise catecholamine control of ion channels in β -cells of these knockout mice remains to be defined (120). An additional channel that may be involved, but has not yet been shown as a target for norepinephrine, is a voltage-gated K^+ (Kv) channel that is activated by ghrelin via $G\alpha_{i2}$ (17). Considering these effects of catecholamines on the β -cell membrane potential, it is clear that they hyperpolarize the cell in a PTX-sensitive manner, but the extent to which the individual K^+ channels are involved may vary in importance depending on the species and the conditions. The effect of the hyperpolarization of course is to eliminate or reduce the activation of voltage-dependent Ca^{2+} channels, Ca^{2+} entry into the cell, and stimulation of insulin secretion. That the effects of hyperpolarization are complicated was shown in a study of β -cells that exhibited oscillations of $[Ca^{2+}]_i$ in response to glucose stimulation and β -cells that did not (101). Norepinephrine had different effects on these cells. In nonoscillators, norepinephrine caused a simple decrease in $[Ca^{2+}]_i$. In oscillators, norepinephrine decreased the amplitude and frequency of the oscillations. Furthermore, the onset of the effect of norepinephrine was immediate in nonoscillators but not always so in oscillators. It was

shown that these different effects of norepinephrine were not due to any effect on Ca^{2+} channels but solely on K^+ channels.

Subsequent to the finding that PTX-sensitive G proteins were involved in the control of channel activity, investigators sought to determine which G proteins and corresponding subunits were involved. The first approach was by Ribalet and Eddlestone (95), who showed that α -subunits of Gi/Go proteins activated K_{ATP} channels in HIT-T15 and RINm5F β -cell lines. While their experiments indicated that the Gi/Go proteins were likely acting directly on the channels, an indirect action could not be ruled out. For instance, a membrane-delimited sequence of reactions downstream of the G proteins could be involved. That neither changes in ATP or cyclic AMP are involved can be seen from patch-clamp experiments performed under whole cell conditions when ATP and cyclic AMP concentrations were maintained constant in the pipette solution. Under these conditions, norepinephrine still activated the K_{ATP} channels in 832/13 cells (137). While other signaling moieties could be involved, the effect of norepinephrine is membrane-delimited because with the outside-out membrane patch configuration in the presence of ATP_i the open activity of the K_{ATP} channels (NP_o) was doubled without any change in conductance (95, 137). Other approaches to determine the identity of the G proteins mediating the effects of inhibitors of insulin secretion on the K_{ATP} channel include the use of antibodies and blocking peptides directed against the G protein subunits (137). In the case of antibodies diffused into the cell via the pipette under whole cell conditions, a common anti-G β had no effect, while a common anti-G α (against Gi,o,t,z,gust) effectively inhibited the effects of norepinephrine on membrane potential and the K_{ATP} channel (137). Following up on this, specific antibodies against various individual G protein α subunits were used. The results were intriguing because a combination of Gi and Go proteins appears to be required for the full effect of norepinephrine on the channel. Applying antibodies to either Gi or Go alone gave only partial inhibition of the norepinephrine action. According to these data, activation of K_{ATP} channels by norepinephrine requires $G\alpha_{i1}$ and/or $G\alpha_{i2}$ and $G\alpha_{o2}$ proteins (137). This surprising conclusion was confirmed by studies with blocking peptides mimicking the COOH termini of the α -subunits. Applying either one of the corresponding $G\alpha_{i1/2}/G\alpha_{o2}$ blocking peptides only partially inhibited the action of norepinephrine while the application of both was fully effective. Blocking peptides for $G\alpha_{i3}$ and $G\alpha_{o1}$ had no effect on the action of norepinephrine. Thus activation of K_{ATP} channels by norepinephrine requires $G\alpha_{i1}$ and/or $G\alpha_{i2}$ and $G\alpha_{o2}$ (137). The lack of definition at present regarding $G\alpha_{i1}$ and $G\alpha_{i2}$ is due to the fact that the antibodies and peptides that block the G protein receptor interactions are directed against the COOH termini of the α -subunits of the G proteins where $G\alpha_{i1}$ and $G\alpha_{i2}$ have the same amino acid sequence. Major questions to be answered now are why both Gi and Go are required and where they bind to produce their effects.

Inhibition of Adenylyl Cyclases

While there has been little research published over the past few years on the effects of norepinephrine on the activity of adenylyl cyclases in the β -cell, for completeness a brief account of the effects of norepinephrine and other inhibitors on the enzymes in the β -cell is presented here. Since the paper by

Samols et al. (100) in 1965 showing that glucagon has a potentiating effect on glucose-stimulated insulin secretion, it has been clear that adenylyl cyclases and cyclic AMP are playing major roles in insulin secretion, β -cell function, and metabolic homeostasis. This observation has been succeeded by many reports on the potentiating effects of cyclic AMP via peptides such as glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic peptide (GIP) originally known as gastric inhibitory polypeptide, vasoactive intestinal peptide (VIP), pituitary adenylyl cyclase activating peptide (PACAP), and others. The effects of these peptides to potentiate stimulated insulin secretion is blocked or reduced by the effect of norepinephrine to inhibit adenylyl cyclases. The importance of these peptides is emphasized by the fact that knowledge of the effects of GLP-1 has been transformed into therapy for type 2 diabetes by the development of long-acting GLP-1 analogs and slow-release formulations, and by inhibitors of dipeptidyl peptidase 4 (DPP-4), the enzyme that rapidly breaks down GLP-1 in the body (2, 24).

Cyclic AMP in the β -cell is elevated by numerous agonists, as just described, and also by glucose (23). Its effects are to potentiate insulin secretion and to stimulate and modify gene expression controlling multiple functions. Prominent among these are insulin biosynthesis, replication, and apoptosis (23). Mediators of these effects are protein kinase A (PKA), the exchange proteins activated by cyclic AMP (Epac), and cyclic AMP response elements and other proteins in the nucleus. Consequently, norepinephrine and other inhibitors of adenylyl cyclases that decrease cyclic AMP levels also have multiple effects on β -cell function. Of the nine known isoforms of the trans-membrane adenylyl cyclases (AC I–AC IX), the following have been reported present in β -cells: AC I and VIII (93); AC V and VI (66); AC I, VI, and VIII (14); and ACs I–IV, VI, and VIII (32). There is also a soluble adenylyl cyclase (sAC) (11). The latter is distinct in that it is not controlled by heterotrimeric G proteins but by intracellular bicarbonate, calcium, and ATP (11). The Ca^{2+} -activated adenylyl cyclases, ACs I, III, and VIII, are likely responsible for the elevation of cyclic AMP by glucose. It is not known which isoforms of adenylyl cyclase in the β -cell are inhibited by norepinephrine. However, as is the case for all the effects of norepinephrine to inhibit insulin secretion, the inhibition is mediated by the PTX-sensitive heterotrimeric Gi/Go proteins (61). While information on the individual isoforms of Gi/Go proteins that mediate the inhibition of the ACs is incomplete, it is known that $\text{G}\alpha_{i2}$ and $\text{G}\alpha_{i3}$ proteins mediate the inhibition of AC by galanin in the RINm5F cell (75). Future studies should focus on which individual Gi/Go protein isoforms inhibit which individual isoforms of the adenylyl cyclase family. More speculative would be to determine whether norepinephrine interferes in any way with the formation of multiprotein scaffolding proteins such as the A kinase anchoring proteins that play roles both upstream and downstream of cyclic AMP elevation (13, 15, 47, 88).

The Mechanism of Action of Norepinephrine on Exocytosis per se (the so-called Distal Site)

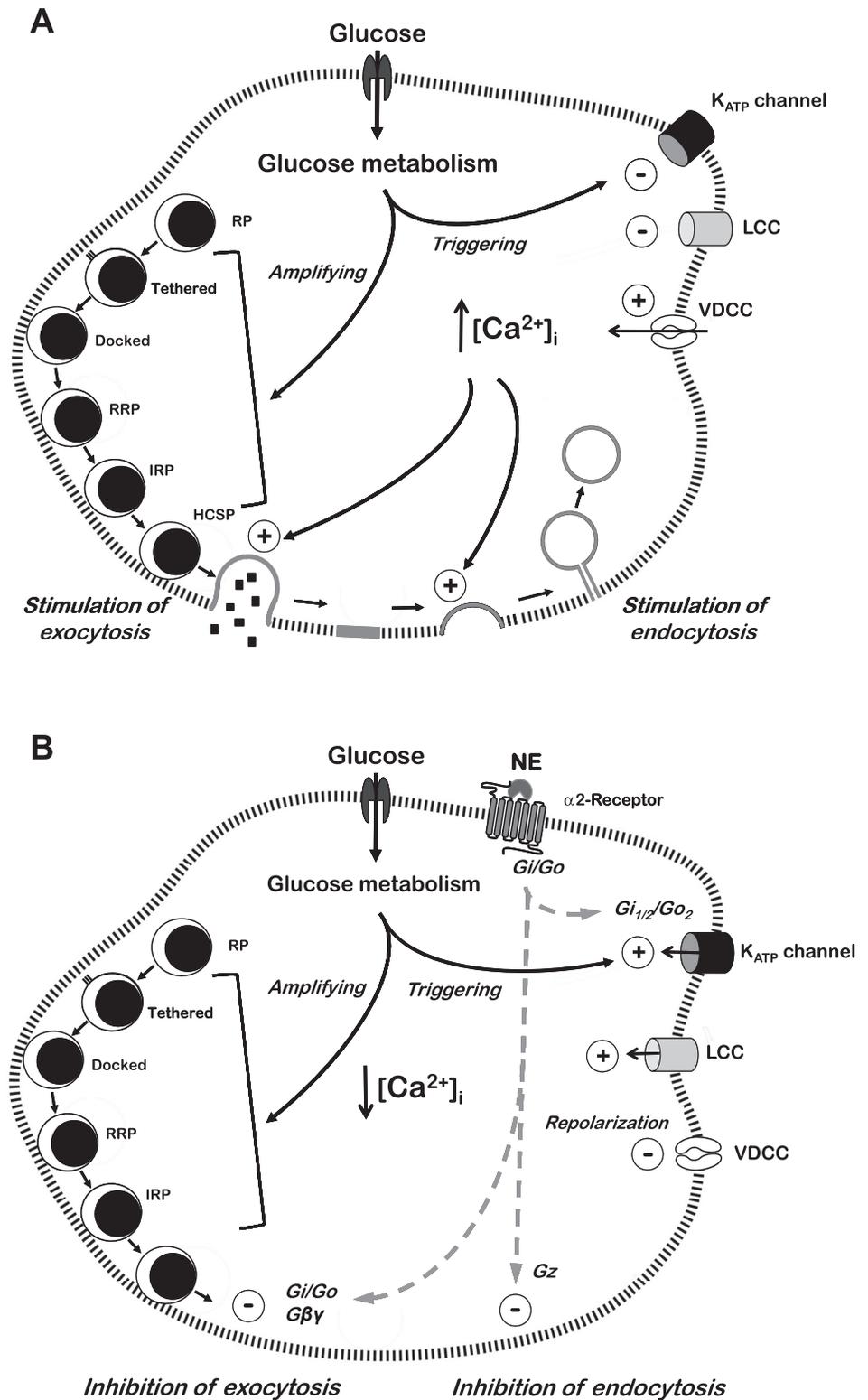
An indication that physiological inhibition of insulin secretion could be effected at a distal site was seen as early as 1977 (129) and with further evidence provided later (1, 46, 76, 105,

124). However, more than three decades would go by before the mechanism was understood and the G protein $\beta\gamma$ subunit identified as the mediator of the inhibition by an effect to block the interaction of the Ca^{2+} -sensor synaptotagmin with the proteins involved in exocytosis (7). In the β -cell, these proteins, the SNARE proteins, include the t-SNAREs synaptosomal-associated proteins of 23 kDa (SNAP-23) and 25 kDa (SNAP-25), syntaxin isoforms at the plasma membrane, and the v-SNAREs synaptobrevin-2 (also known as vesicle-associated membrane protein-2 or VAMP-2) and cellubrevin (VAMP-3) at the granule membrane. Ca^{2+} -stimulated exocytosis also involves the Ca^{2+} -sensor synaptotagmin present in the granule membrane, Munc-18, and several other proteins. For reviews on this topic see references 45 and 116. A minimal description of the mechanism of exocytosis in response to glucose in the β -cell would include a rise in $[\text{Ca}^{2+}]_i$ and Ca^{2+} binding to the synaptotagmins VII and IX (25). Subsequent synaptotagmin binding to SNARE complexes, comprising SNAP-23 and SNAP-25, syntaxin 1A and syntaxin 4, and VAMP-2, with the involvement of Munc-18c and other proteins initiates granule membrane/plasma membrane fusion, exocytosis, and the release of insulin. Understanding of the mechanism of the distal inhibitory effect in the β -cell came from studies on neuronal cells on the inhibition of neurotransmitter release downstream of elevated $[\text{Ca}^{2+}]_i$ by serotonin. It was shown that the inhibitory G protein $\beta\gamma$ subunit bound to the SNARE complex at the COOH terminus of SNAP-25. This competitively blocks the interaction between synaptotagmin and SNAP-25 and inhibits exocytosis (7, 8, 27). Furthermore, a high Ca^{2+} concentration enabled the Ca^{2+} -activated synaptotagmin to compete successfully with $\text{G}\beta\gamma$ for SNARE protein binding and overcome the inhibitory effect. Similar studies to these confirmed that this mechanism was operating in the β -cell (135). Thus, antibodies against $\text{G}\beta$ blocked the inhibition of exocytosis by norepinephrine. The $\beta\gamma$ -activating peptide mSIRK (31) inhibited exocytosis, and when norepinephrine and mSIRK were applied together there was no additional inhibition relative to the two applied singly. The inhibitory effects of both norepinephrine and mSIRK were overcome by a high Ca^{2+} concentration. Additionally, botulinum toxin A, which cleaves off a portion of the COOH terminus of SNAP-25, blocked the inhibition of exocytosis by norepinephrine. A COOH-terminal blocking peptide of SNAP-25 also prevented the inhibitory effect. Thus, the distal inhibition of exocytosis by norepinephrine in the β -cell is due to $\text{G}\beta\gamma$ binding to the SNARE complex, inhibition of synaptotagmin binding, and blockade of SNARE protein function (135). Additionally, it was found that the distal inhibition by norepinephrine is due to a decrease in the number of exocytotic events with no change in vesicle size or fusion pore properties (135). This confirms that the inhibition occurs at a site common to the mechanisms that trigger exocytosis following an increase in $[\text{Ca}^{2+}]_i$. These features of glucose stimulation and norepinephrine inhibition are shown in Fig. 1.

Norepinephrine Has Two New Tricks and a New G Protein Partner

Norepinephrine retards the refilling of the readily releasable pool of insulin containing granules. Secretory cell granule pools such as the large reserve pool (RP), the readily releasable pool (RRP)

Fig. 1. A: stimulation of insulin secretion by glucose. The metabolism of glucose stimulates insulin release by closure of ATP-sensitive K^+ (K_{ATP}) channels and a low-conductance K^+ channel (LCC), depolarization of the cell, and elevation of intracellular Ca^{2+} . This is referred to as activation of the triggering pathway. The amplifying pathway is increasingly activated by glucose metabolism over time by the buildup of an as yet unknown signal or signals from the mitochondria, in conjunction with the still elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). This pathway increases the flow of granules from the reserve pool (RP) to the immediately releasable state where, in an activated cell, they undergo exocytosis. The two pathways are considered responsible for the first and second phases of glucose-stimulated insulin secretion, respectively. The elevated $[Ca^{2+}]_i$ also initiates the necessary compensatory endocytosis. RRP, readily releasable pool; IRP, immediately releasable pool; HCSP, highly Ca^{2+} -sensitive pool; VDCC, voltage-dependent calcium channel. B: norepinephrine (NE)-induced inhibition of glucose-stimulated insulin secretion and endocytosis. NE inhibits insulin release in two ways, both of which involve the pertussis-sensitive heterotrimeric G_i and G_o proteins. In one, NE opens the K_{ATP} channels to hyperpolarize the cell, and in this mechanism, $G_{i/2}$ and G_{o2} are required for the full effect on the channels. In the other, NE inhibits release by the effect of $G\beta\gamma$ (derived from G_i and/or G_o) to directly inhibit the SNARE protein involvement in exocytosis. NE inhibits endocytosis via activation of G_z (see text).



(6, 26, 40, 80, 114, 115), the immediately releasable pool (IRP) (6, 26, 80, 115), and the highly Ca^{2+} -sensitive pool (HCSP) (40), have to be carefully defined. The reason being that their names stem from studies in various cell types with different patterns of exocytosis and different control mechanisms, because of different circumstances in the same cell type, e.g., primed or not primed in the β -cell, and because of the different

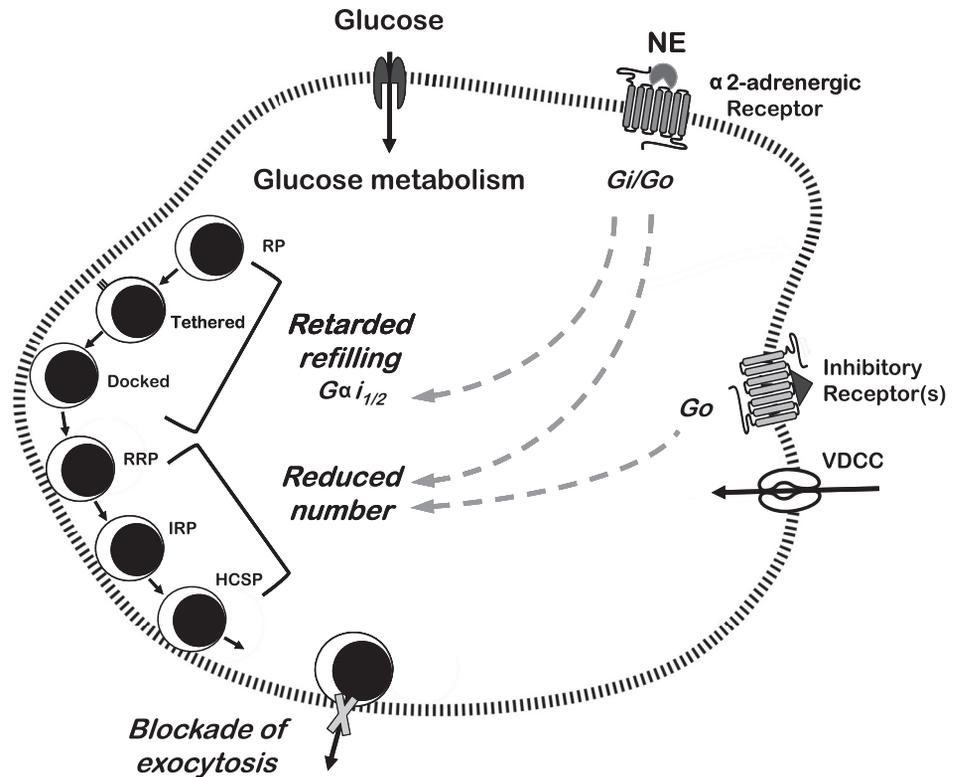
experimental protocols under which they have been studied. The need for careful definition is further emphasized by the fact that different researchers have used the terms IRP and RRP to describe the same pool. We (114, 115) and others (6, 26, 77) have described both an IRP and an RRP in the β -cell similar to the terminology for other cell types (29, 125) and as originally proposed in chromaffin cells (39). In this terminology, the IRP

is immediately released when the cell is stimulated. Subsequently, if the stimulation is prolonged, the IRP has to be refilled from the RRP, and the RRP from the RP in order for the flow of exocytosis to continue. The IRP is equated with the first phase of glucose-stimulated insulin release, and the size of the first phase response will be an indication of the number of granules in the IRP. Of interest here is the time course of the IRP release and its relationship to the first phase of glucose-stimulated insulin release. Under conditions where exocytosis from a single cell is measured by capacitance change during depolarization, the IRP is released in milliseconds (135, 136). In the case of glucose stimulation of perfused isolated islets or perfused pancreas, the release occurs over a first phase period of ~ 8 min (82, 113–115). Why then is the IRP measured on single cells equated with the first phase of insulin release? The strongest evidence is that the number of granules released per cell is similar (6, 115), and the most likely explanation for the different time course is that the depolarized single cell is activated in milliseconds and the exocytosis recorded by capacitance change in milliseconds. Cells in the intact islet are activated at different times over the 8-min period because glucose has to diffuse into the tissue and be metabolized before stimulating the cell, and then the insulin has to diffuse out before it can be measured. The rate of conversion of granules from the RRP to the IRP will determine the rate of the second phase response (115). For the purpose of this review the term RRP is also defined operationally as the pool or combination of pools from which granules are rapidly released in response to an extremely large Ca^{2+} stimulus under patch-clamp conditions. Furthermore, this stimulus depletes the pool so that a subsequent stimulation shortly after the first induces only a minimal response. This operational definition is used because the studies that defined the effect of norepinephrine to retard the refilling process used a large Ca^{2+} stimulus that depleted the pool and also prevented any inhibitory effects of norepinephrine on exocytosis. This definition of the RRP most likely encompasses all three releasable pools described thus far in the β -cell literature, the RRP, IRP, and HCSP. The effect of norepinephrine to inhibit the refilling of the RRP was discovered during capacitance studies to determine which G proteins were involved in the distal inhibition of exocytosis (135). Under whole cell conditions, an antibody against $\text{G}\beta$ largely eliminated the inhibitory effect of norepinephrine on exocytosis when a single depolarizing pulse was applied to the cell, while an antibody common to several $\text{G}\alpha$ subunits ($\text{G}\alpha_{\text{o,t,z,gust}}$) was without effect. This was consistent with the concept that it is the $\beta\gamma$ subunit that mediates the distal inhibitory effect. However, when the experiments were performed with a series of consecutive stimulations, the antibody against $\text{G}\beta$ was fully effective against the first stimulation but appeared to become less effective over time (135). A possible explanation for this is that norepinephrine, in addition to blocking exocytosis, had an effect to reduce the rate of refilling of the RRP. Thus the antibody against $\beta\gamma$ was indeed completely blocking the exocytosis that was occurring over time, but the number of granules available for exocytosis was progressively less relative to the control cells. This was confirmed by using a two-pulse protocol to measure refilling rates directly. A depolarizing pulse providing a high Ca^{2+} -influx over 500 ms was applied to deplete the RRP and to block the effect of norepinephrine on exocytosis. Subsequent second pulses of the same

magnitude were then applied after time intervals of 1 to 40 s. The size of the exocytotic responses to the second pulse at each time point estimates the rate at which the RRP is being refilled after depletion. Under the conditions imposed, control cells were 40% refilled 2 s after the first stimulation and 80% after 30 s. In contrast, cells exposed to norepinephrine were only 10% refilled after 2 s and $<40\%$ after 30 s. Subsequently, $\text{G}\alpha_{\text{i}_1}$ and/or $\text{G}\alpha_{\text{i}_2}$ were found to be the mediators of the effect (135) (Fig. 2).

In seeking the mechanism by which norepinephrine slows the refilling process, all the steps involved must be considered. Thus translocation of granules from the RP to the plasma membrane, tethering, docking with the membrane, and priming for release (either biochemical, locational or both) all have the potential to be rate limiting. Note that these steps apply to both the conventional ideas of granule docking and being prepared for release at docked sites on the membrane to the more recent idea of “newcomers” moving rapidly from the RP to exocytosis (84, 85). From capacitance studies it was concluded that the rate of refilling of the RRP is dependent on the metabolism of glucose in the absence of any changes in Ca^{2+} influx (22, 94). A change in cellular ATP levels was identified as one possible reason for this, but other signals derived from glucose metabolism are not ruled out. Activation of protein kinase C (PKC) also increases the size of a highly Ca^{2+} -sensitive vesicle pool (134). Treatment of cells with phorbol esters, which activate both PKC and diacylglycerol (DAG)-binding proteins, increases both the size and refilling rate of RRP (29, 64, 109, 112, 126, 130) and potentiates insulin secretion by increasing the total number of vesicles that are available for release. The DAG-binding protein Munc-13 (10) has a role in priming vesicles and increasing RRP pool size (64). Consequently, it is likely that the effects of phorbol esters are mainly due to activation of Munc-13. Direct interactions of PKA and PKC with the secretory machinery have also been suggested in other cell types, such as chromaffin cells and hippocampal neurons, where the size of the RRP and its rate of replenishment is increased (109, 112). While inhibition of DAG-binding proteins and PKC could be involved in the retarding effect of norepinephrine, there is no evidence in the β -cell literature for an inhibitory effect of norepinephrine on DAG-binding proteins or PKC. PKA-dependent and Epac-dependent effects of cyclic AMP on RRP refilling and size are known and have been extensively reviewed (36, 37, 48, 51, 103). Epac signals through the low-molecular-weight G protein Rap1 (106). Despite these data, the well-characterized effect of norepinephrine to inhibit adenylyl cyclases and lower cyclic AMP levels is not responsible for the retarded refilling of the RRP because the patch-clamp experiments in which the effect was demonstrated were buffered with a maximally effective concentration of cyclic AMP in the intracellular (pipette) solution (135). Nevertheless, low-molecular-weight G proteins have been implicated in the control of granule translocation, tethering, docking, and the size and refilling of the rapidly releasable pool. They will be mentioned here only briefly for two reasons: 1) their roles in the control of insulin secretion have been comprehensively reviewed recently (62, 128) and 2) as yet there is no apparent mechanism to connect them to an effect of norepinephrine via $\text{G}\alpha_{\text{i}_{1/2}}$ to retard the refilling of the pool. Despite this, given that heterotrimeric G proteins do signal to low-molecular-weight G proteins (70, 123), a connection may

Fig. 2. Effect of NE to retard the refilling of the RRP and reduce the number of docked granules. NE slows the refilling of the RRP via $G\alpha_{i/2}$. The site of action is unknown and could be at any point or multiple points between the RP and the RRP. Overexpression of the α_2 -adrenergic receptor decreases the number of docked granules (98) as does Go (137).



be found in the future. It should be noted that some of the implications that the low-molecular-weight G proteins affect one or other of the various steps between translocation and exocytosis are derived from indirect evidence. An effect of a low-molecular-weight G protein on the first phase of glucose-stimulated insulin release or on a response to a depolarizing concentration of KCl has been interpreted as an effect on the size of the RRP even though the pool size may not have been measured directly. An effect on the second phase of release has been interpreted as an effect on the rate of refilling of the RRP because the RRP has to be refilled from the RP in order for the flow to the IRP and exocytosis to continue. This includes several factors, including the rates of granule translocation, tethering, docking, and priming. Only when these events are measured individually will there be any certainty as to the precise targets and actions of the low-molecular-weight G proteins. With that proviso, there are reports that low-molecular-weight G proteins affect all these necessary aspects of secretion. Examples include RalA (67, 68), Rab3a (77), and Rab27a (30, 49, 50, 77, 132), all three of which are associated with tethering and docking. Priming and pool size are reportedly controlled by Rap1 (106), RalA (68), and Rab3a (77, 131); exocytosis by Rab3a (77) and Rab11 (117); and the refilling of the pool after exocytosis by Rab27a (30, 49) and RalA (68). The effect of the α_2 -adrenergic receptor (99) and the effect of $G\alpha_o$ (138) on the number of docked granules is discussed later in this review (Fig. 3).

The IRP in the β -cell that is released by glucose stimulation during the first phase of insulin secretion (114, 115) is a subset of the RRP and is of great importance for at least three reasons: 1) a diminished first phase response is a feature of type 2 diabetes and occurs even before the onset of overt symptoms of the disease (19, 28, 60); 2) in glucose-induced biphasic insulin

secretion, the size of the IRP determines the size of the first phase; and 3) the rate of refilling of the IRP after the first phase of release is an effector of the glucose-amplifying pathway and determines the rate and magnitude of the second phase response (80, 114, 115). It does this by time-dependently accelerating a rate-limiting step in the refilling of the IRP to induce the rise to the second phase plateau—at which point the conversion rate of granules from the RRP to the IRP is maximal and equal to the rate of exocytosis (114, 115). Physiologically, it is of interest now that norepinephrine inhibits both of the major pathways by which glucose induces biphasic insulin secretion. The first phase of glucose-stimulated release is due to the K_{ATP} channel-dependent or “triggering” pathway that involves closure of the K_{ATP} channels, depolarization of the β -cell, increased Ca^{2+} influx, and increased $[Ca^{2+}]_i$. This is blocked by the effect of norepinephrine to activate the K_{ATP} channels and thereby hyperpolarize the cell (104). The second phase of release is due to the K^+ channel-independent or “amplifying” pathway and is caused, as just described, by an increased rate of refilling of the IRP (114, 115). This refilling is restrained by norepinephrine. The mechanism of action of $G\alpha_{i/2}$ to slow the refilling of the RRP remains to be worked out but is also important as a future potential therapeutic target, e.g., for the reduction of insulin release in the various forms of hyperinsulinism (20).

Norepinephrine inhibits endocytosis via activation of G_z . Recently, it was found that norepinephrine exerted an inhibitory effect on endocytosis (136). This finding was novel but could have been anticipated as both exocytosis and endocytosis are stimulated by increased $[Ca^{2+}]_i$. Therefore, as norepinephrine can inhibit exocytosis, even in the face of increased $[Ca^{2+}]_i$, endocytosis would not be needed and must be prevented. An example of such a situation would be stimulation of

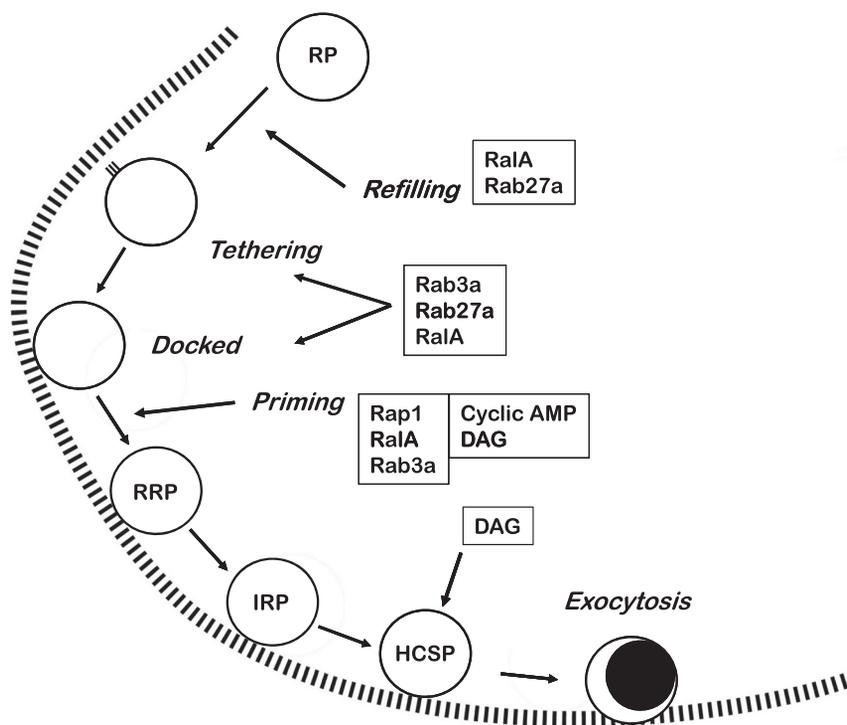


Fig. 3. Low-molecular-weight G proteins and second messengers that are involved in the control of granule movement, number, and preparedness for exocytosis. Important players shown here are RalA, Rab3a, Rab27a, Rap1, cyclic AMP [via PKA and exchange proteins activated by cyclic AMP (Epac)], and diacylglycerol (DAG) (via PKC and DAG-binding proteins). All are involved in controlling the number of granules in the RRP. Their actions and references to them are given in the main text.

insulin release by arginine after a high-protein meal. Norepinephrine would inhibit exocytosis but would not inhibit the arginine-induced increase in $[Ca^{2+}]_i$. In this way, the two events, inhibition of exocytosis and inhibition of endocytosis, are coordinated. Also completely novel was the finding that the PTX-insensitive heterotrimeric G protein Gz mediates the effect. While Gz has not previously been linked to norepinephrine, it is present in the β -cell and is activated by PGE₁ (57, 58). The probable reason why this effect of norepinephrine has been hidden for so long is that as norepinephrine inhibits exocytosis, there was little need to look for an effect on endocytosis. Only when the effect of norepinephrine to inhibit exocytosis was blocked by high Ca^{2+} influx was the effect on endocytosis apparent (135, 136). Evidence that normal control of endocytosis is important for the β -cell is as follows. 1) Cyclosporine and tacrolimus, powerful inhibitors of calcineurin and consequently endocytosis, are used extensively after organ transplantation and are associated with posttransplant diabetes mellitus. Furthermore, while insulin resistance plays a role in the development of posttransplant diabetes mellitus, reduced insulin secretion appears to have the major role (34). 2) Tacrolimus inhibits insulin gene expression, insulin mRNA levels, and insulin secretion (92). 3) The dominant interfering dynamin mutant DynK44A or siRNA knock-down of dynamin inhibit insulin release (79). While the mechanism(s) involved in the development of posttransplant diabetes mellitus are not known in detail, and are likely multifaceted, the inhibition of endocytosis by cyclosporine and tacrolimus and the resultant deleterious effects on insulin secretion should certainly be subject to serious investigation. Endocytosis, first documented in the β -cell by Orci et al. in 1973 (87), has received considerable interest in the past decade (35, 42, 59, 63, 71, 72, 81, 86, 122). The term applies to several mechanisms that exist to remove cell membrane components. Among these are receptor internalization, phagocytic pro-

cesses, retrieval of plasma membrane constituents for renewal and, of direct relevance to the β -cell, the endocytosis of vesicles, vesicle proteins, lipids, and other constituents after exocytosis, i.e., compensatory endocytosis. In general, there are five possible ways in which granules can undergo endocytosis (96): 1) "kiss and run" retrieval; 2) the collapse of a granule and its retrieval by clathrin-mediated endocytosis; 3) bulk retrieval of membrane components; 4) the collapse of a granule followed by dispersal into patches of membrane that are subsequently retrieved; and 5) the collapse of a granule followed by complete dispersal of the granule membrane and retrieval of the individual components (96). Obviously, distinct mechanisms will be involved to facilitate these disparate processes. Therefore, important to understanding the endocytosis that follows exocytosis in the β -cell are the types of exocytosis taking place. Whether exocytosis occurs by full fusion of the granules or partial fusion (kiss and run) is critical, because the former requires retrieval of granule membrane components from the plasma membrane and the latter requires retrieval of the essentially intact granule membrane. The extent to which the two processes occur is a matter of debate (71, 73), but the evidence in favor of the simultaneous operation of both mechanisms in the β -cell is strong. Endocytosis is stimulated by increased $[Ca^{2+}]_i$ in β -cells (21, 91) as in other cells and occurs in two phases, similar to the two phases of endocytosis in chromaffin cells (110). Increased $[Ca^{2+}]_i$ sets in motion a series of reactions beginning with the activation of calcineurin, dephosphorylation of the dephosphins, and the subsequent involvement of dynamin (12, 69, 108) and other players. Calcineurin inhibitors such as deltamethrin, tacrolimus, and cyclosporin A block the initiating step in the sequence of events that lead to Ca^{2+} -induced endocytosis. As a result, they block endocytosis by reducing the number of events without any effects on later steps such as vesicle size, fission kinetics, or other aspects of the mechanisms involved. Norepinephrine

also inhibits endocytosis by reducing the frequency of endocytotic events and without changing the size of the vesicles. However, unlike the calcineurin inhibitors, it does affect the kinetics and Gz is assumed to act at a late stage of endocytosis (136). This action of norepinephrine to inhibit endocytosis is novel, as is its activation of Gz. However, it is likely that this effect will be found in many other cells in which agonists inhibit exocytosis downstream of elevated $[Ca^{2+}]_i$. Two such agonists are serotonin (7, 8, 27) and endothelin (3).

The Roles of Go in the Control of Insulin Release

Regulation of the number of docked granules. Of related interest to the effect of norepinephrine to slow the rate of refilling of the RRP are two recent findings. The first is that humans, with a single-nucleotide polymorphism in the α_{2A} -adrenergic receptor gene who exhibit overexpression of α_{2A} -adrenergic receptors with reduced insulin secretion and increased risk of type 2 diabetes, had a decrease in the number of docked β -cell granules (99). This study is discussed in more detail in the Clinical Implications section of this review, which deals with the potential clinical implications of the α_2 -adrenergic receptors. The second finding is that $G\alpha_o$ has a controlling influence on the number of docked granules in the β -cell (138). In this study, the authors used a tissue-specific β -cell knockout of $G\alpha_o$ in the mouse and studied granule docking with transmission electron microscopy (TEM), with quantitative morphometry, and with total internal reflection fluorescence microscopy (TIRFM). They found that the number of docked granules in the β -cell was significantly increased relative to wild-type controls, indicating that $G\alpha_o$ maintains a suppressive effect on the number of docked granules. With TEM, the number of docked granules, defined as those in contact with the plasma membrane, was twice that of the wild type. Using TIRFM, the number of docked granules was increased by one third. TEM and TIRFM detect docked granules differently, but regardless of this both techniques detected a significant increase in the number of docked granules in the β -cells of the $G\alpha_o^{-/-}$ mutant mice. In further studies, no differences were detected in granule trafficking or in the numbers of "newcomer granules" in the absence of $G\alpha_o$ in the β -cell. It is clear from this study and others that control over the refilling rate and the number of granules in the RRP is a complex of several overlapping functions.

The mechanism underlying the name "islet activating protein." PTX increases plasma insulin levels and enhances stimulated insulin secretion. It was because of these findings that it was first named islet-activating protein (53, 121). The reasons for these tonic and enhancing effects have remained unknown until recently, but now two explanations have been put forward. In the first, it is proposed that ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (41), is responsible. While ghrelin is produced mainly in the stomach (5), it is present in the islet (17). There are several lines of evidence in favor of the idea that ghrelin is responsible for the islet-activating effect of PTX. Ghrelin inhibits insulin secretion in vivo and in vitro (18). There is an inverse relationship between fasting insulin levels and ghrelin concentrations in blood (17, 18). In addition, antiserum against ghrelin increases insulin release and lastly, PTX blocks the effects of ghrelin (18). Antisense oligonucleotides against $G\alpha_i_2$, but not

those against $G\alpha_i_1$ or $G\alpha_i_3$, block the effect of ghrelin to inhibit glucose-stimulated insulin release and identify $G\alpha_i_2$ as the G protein mediator of the effects of ghrelin. Ghrelin gene knockout blocked the insulin release-enhancing effect of PTX by 70–80%, indicating that much of the islet-activating effect of PTX was due to ghrelin (17, 18). In the second, more recent explanation, it is reported that $G\alpha_o_2$ is responsible for the tonic inhibition that is lifted by PTX. Mice lacking $G\alpha_o_2$ but not those lacking $G\alpha_o_1$ or any of the Gi subunits handle glucose loads more efficiently than wild-type mice and do so by increased insulin release (127). In this study, the authors generated $G\alpha_o_1$ and $G\alpha_o_2$ knockout mice. In glucose tolerance tests using these mice and the wild-type controls, the responses of wild-type and $G\alpha_o_1^{-/-}$ mice were similar. However, the response of the $G\alpha_o_2^{-/-}$ mice was much different, with a lower peak glucose level and more rapid decline to baseline values. As normalized insulin tolerance tests were similar in wild-type and the $G\alpha_o_2^{-/-}$ mice, it was concluded that the difference in the glucose tolerance tests was likely due to differences in insulin secretion, with the $G\alpha_o_2^{-/-}$ mice secreting more insulin in response to glucose. This was confirmed by experiments on isolated islets from these mice. It was further shown that somatostatin failed to inhibit insulin secretion in the $G\alpha_o_2^{-/-}$ mice. Norepinephrine was not tested. It remains to be seen how these two convincing explanations for islet activation by PTX can be reconciled. In one, the activation is due to $G\alpha_i_2$ in response to endogenous ghrelin, and in the other, $G\alpha_o_2$ is acting either constitutively or in response to hormones that inhibit insulin secretion via $G\alpha_o_2$. While both mechanisms are likely to be involved, further studies will be required to resolve the issue.

Clinical Implications

α_2 -Adrenergic receptor involvement in diabetes. Research into the various causes of type 2 diabetes has usually focused on the combination of insufficient insulin secretion to control blood glucose levels and insulin resistance. Most of the emphasis on the β -cell has been on the mechanisms of stimulation of insulin secretion and how they might be impaired or inadequate. There has been less interest in the physiological inhibitory mechanisms of insulin secretion despite the knowledge that excessive inhibition of insulin secretion could well be a cause of diabetes. An early indication of this was the finding that clonal β -cells overexpressing the α_2 -adrenergic receptor exhibited tonic inhibition of insulin secretion. The authors suggested that "abnormalities in expression or function of such receptors could be a contributory factor in the impaired insulin secretion present in type 2 diabetes" (97). Studies on α_{2A} -adrenergic receptor knockout C57BL/6J mice provided further evidence of receptor-induced tonic inhibition. When these knockout mice were compared with their wild-type controls, they showed lower blood glucose levels and higher plasma insulin levels. Their glucose tolerance was also significantly better than the wild type (111). In an early study on polymorphism of the α_{2A} -adrenergic receptor, genomic DNA was isolated from 147 hypertensive patients. Genotypes at the α_{2A} -adrenergic receptor were identified and studied in relation to hypertension, and lipid and glucose metabolism (78). While no association of α_{2A} -adrenergic receptor polymorphism with hypertension was found, there was a significant reduction in

the amounts of hemoglobin A_{1c}, hemoglobin A_{1c}, and total cholesterol in blood from patients with the D allele of the receptor. Similar trends were seen for glucose, triglycerides, and low-density lipoprotein cholesterol, but they failed to achieve statistical significance. The data from this polymorphism study did not suggest that it confers an increased risk of diabetes. Nevertheless, these and other genetic studies (9) stress the importance of reevaluating the α_{2A} -adrenergic receptor in the causation of some forms of diabetes. This is especially important since, more recently, overexpression of α_{2A} -adrenergic receptors was shown to contribute to type 2 diabetes (99). In the Goto-Kakizaki (GK) rat, with its diabetes susceptibility locus that includes the α_{2A} -adrenergic receptor gene, there was a decrease in the number of docked granules, as estimated by quantitative morphometric analysis, associated with reduced insulin secretion. Blockade of the α_{2A} -adrenergic receptor by yohimbine reversed the defects so that normal function was restored. This identified the defect at the level of the α_{2A} -adrenergic receptor. When variants of the human α_{2A} -adrenergic receptor gene were studied, polymorphisms around the α_{2A} -adrenergic receptor were connected to decreased insulin secretion and increased risk of type 2 diabetes. In vitro studies with human islets confirmed that islets from humans with the risk-carrying α_{2A} -adrenergic receptor polymorphism had increased numbers of the α_{2A} -adrenergic receptors, lower numbers of docked granules, and reduced insulin secretion in response to glucose stimulation. These were reversed by α_{2A} -adrenergic receptor blockade. It seems likely that the reduced docking and insulin secretion result from the overexpressed α_{2A} -adrenergic receptors.

Summary

The actions of norepinephrine on the pancreatic β -cell include activation of K⁺ channels, inhibition of adenylyl cyclases, direct inhibition of exocytosis, retardation of the refilling of the RRP, and inhibition of endocytosis. Only the latter effect of norepinephrine, mediated by G_z, is not via the PTX-sensitive Gi and Go proteins. By virtue of its effects on K⁺ channels and the RRP, norepinephrine inhibits the two major pathways involved in glucose-stimulus secretion coupling (114), the triggering and amplifying pathways, respectively. Its effect to inhibit exocytosis of course blocks both pathways. Augmentation of stimulated insulin secretion by agonists such as GLP-1, GIP, and PACAP is blocked by the effect of norepinephrine to inhibit the activity of adenylyl cyclases. Recently, discovered effects of Go proteins include a suppressive effect of G α_o on the number of docked granules, and a tonic inhibition of insulin release by G α_o . Overexpression of the α_{2A} -adrenergic receptor leads to reduced insulin secretion and a form of type 2 diabetes.

Future Perspectives

Despite decades of work that have uncovered the physiological effects of norepinephrine, much remains to be done. In the clinical area it would seem that polymorphisms of the receptors for all the physiological inhibitors of insulin secretion, for example, somatostatin, ghrelin, and prostaglandins, could be associated with the development of type 2 diabetes. The possible role of inhibited endocytosis in posttransplant diabetes mellitus is also an area for study. The mechanism by which

norepinephrine and presumably other inhibitors retard the refilling of the RRP needs to be understood because it should provide useful targets for the treatment of hyperinsulinism. Are increased expression levels of all the Gi and Go proteins associated with a form of type 2 diabetes? In the area of basic research, we need to investigate the mechanisms of norepinephrine action in greater detail to answer several questions. How are the G proteins and the isoforms of their $\alpha\beta\gamma$ subunits interacting with the effectors of the inhibition of insulin secretion i.e., K⁺ channels, adenylyl cyclases, and the SNARE complexes? Does norepinephrine affect SNARE protein function only in exocytosis and endocytosis or does it affect fusion processes in organelles like the endoplasmic reticulum, Golgi, and lysosomes where other subsets of SNARE proteins are active? What protein(s) does G_z interact with to inhibit endocytosis? Both early and recent findings on the effects of norepinephrine and other inhibitors of insulin secretion indicate the need for further detailed investigation.

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AUTHOR CONTRIBUTIONS

S.G.S. prepared the figures; S.G.S. and G.W.G.S. drafted the manuscript; S.G.S. and G.W.G.S. edited and revised the manuscript; S.G.S. and G.W.G.S. approved the final version of the manuscript.

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