Perspectives in Diabetes

Epac: A New cAMP-Binding Protein in Support of Glucagon-Like Peptide-1 Receptor–Mediated Signal Transduction in the Pancreatic β-Cell

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Recently published studies of islet cell function reveal unexpected features of glucagon-like peptide-1 (GLP-1) receptor–mediated signal transduction in the pancreatic β-cell. Although GLP-1 is established to be a cAMP-elevating agent, these studies demonstrate that protein kinase A (PKA) is not the only cAMP-binding protein by which GLP-1 acts. Instead, an alternative cAMP signaling mechanism has been described, one in which GLP-1 activates cAMP-binding proteins designated as cAMP-regulated guanine nucleotide exchange factors (cAMPGEFs, also known as *Epac*). Two variants of *Epac* (*Epac1* and *Epac2*) are expressed in β-cells, and downregulation of *Epac* function diminishes stimulatory effects of GLP-1 on β-cell Ca^{2+} signaling and insulin secretion. Of particular note are new reports demonstrating that *Epac* couples β-cell cAMP production to the stimulation of fast Ca^{2+}-dependent exocytosis. It is also reported that *Epac* mediates the cAMP-dependent mobilization of Ca^{2+} from intracellular Ca^{2+} stores. This is a process of Ca^{2+}-induced Ca^{2+} release (CICR), and it generates an increase of [Ca^{2+}]$_i$ that may serve as a direct stimulus for mitochondrial ATP production and secretory granule exocytosis. This article summarizes new findings concerning GLP-1 receptor–mediated signal transduction and seeks to define the relative importance of *Epac* and PKA to β-cell stimulus-secretion coupling. *Diabetes* 53:5–13, 2004

T he insulin secretagogue hormone glucagon-like peptide-1 (GLP-1) and its structurally related peptide analogs NN2211, CJC-1131, and ex-endin-4 are potent stimulators of the pancreatic β-cell GLP-1 receptor (GLP-1-R) (1–4). When administered to type 2 diabetic subjects, these peptides exert multiple antidiabetogenic effects: they stimulate insulin secretion, they lower fasting concentrations of blood glucose, and they attenuate the elevation of blood glucose concentration that is typically observed following ingestion of a meal. Such beneficial blood glucose–lowering actions of GLP-1 indicate a usefulness of the hormone as a new therapeutic agent for the treatment of diabetes. Indeed, efforts are now under way to develop synthetic analogs of GLP-1 that exhibit an optimal pharmacokinetic and pharmacodynamic spectrum of action commensurate with their use as therapeutic agents (1).

Simultaneously, efforts have been directed at elucidating the molecular basis for GLP-1-R–mediated signal transduction. Previous studies demonstrate that GLP-1 activates multiple signaling pathways in the β-cell (Fig. 1). These pathways include protein kinase A (PKA), Ca^{2+}/calmodulin-regulated protein kinase (CaMK), mitogen-activated protein kinases (MAPK, ERK1/2), phosphatidyl-inositol 3-kinase (PI-3K), protein kinase B (PKB, *Akt*), and atypical protein kinase C-ζ (PKC-ζ). In addition, evidence exists for actions of GLP-1 mediated by protein phosphatase (calcineurin) and hormone-sensitive lipase. Some of these signaling pathways are likely to play an active role in determining the effectiveness of GLP-1 as a stimulus for pancreatic insulin secretion. They may also confer trophic factor-like actions to GLP-1 that underlie its ability to stimulate β-cell growth, differentiation, and survival (1).

Recent studies of pancreatic β-cell stimulus-secretion coupling reveal the existence of an alternative cAMP signal transduction pathway by which GLP-1 may exert its effects (5–12). GLP-1 is shown to stimulate cAMP production, and the action of cAMP is demonstrated to be mediated not only by PKA, but also by a newly recognized family of cAMP-binding proteins designated as cAMP-regulated guanine nucleotide exchange factors (cAMPGEFs, also known as *Epac*) (13,14). Identification of *Epac* as an intermediary linking the GLP-1-R to the stimulation of insulin secretion has led to an appreciation that the blood glucose–lowering effect of GLP-1 might be reproduced using pharmacological agents that activate cAMPGEFs in a direct and selective manner. Furthermore, the potential interaction of *Epac* with β-cell signaling pathways that subserve stimulatory influences of glucagon, glucose-dependent insulinotropic peptide, or pituitary adenylyl cyclase activating polypeptide is only now becoming apparent.
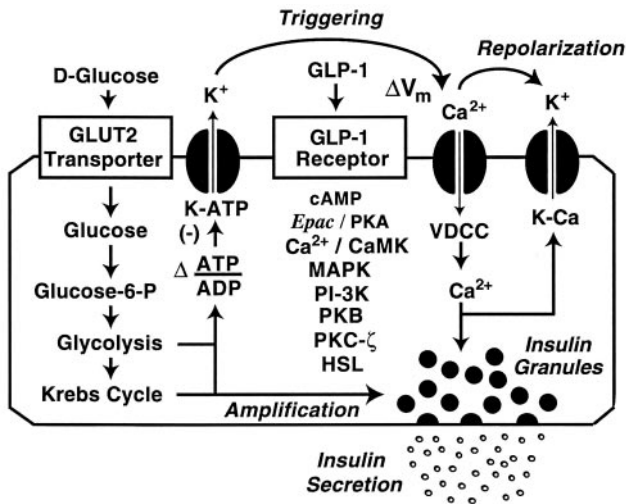
FIG. 1. Regulation of glucose-dependent insulin secretion in the β-cell. Illustrated are the "triggering" and "amplification" pathways by which glucose metabolism leads to insulin secretion (77). Also illustrated are the multiple signal transduction pathways by which GLP-1 influences β-cell function. Uptake of glucose is mediated by the type 2 facilitative glucose transporter (Glut2), and aerobic glycolysis produces an increase of cytosolic [ATP]/[ADP] concentration ratio. This metabolic signal leads to the inhibition of K_{ATP} channels, thereby initiating membrane depolarization (ΔVm), activation of voltage-dependent Ca^{2+} channels (VDCCs), Ca^{2+} influx, and a stimulation of Ca^{2+}-dependent exocytosis (triggering pathway). The opening of K^+ channels (e.g., Ca^{2+}-activated K^+ channels; K-Ca) terminates Ca^{2+} influx by repolarizing the membrane. Metabolism of glucose also generates metabolic coupling factors that act directly at the secretory granules to facilitate their exocytosis (amplification pathway). The function of the triggering and amplification pathways is potentiated by GLP-1 via its effects on glucose-dependent ATP production (12), K_{ATP} channels (52), VDCCs (72), intracellular Ca^{2+} stores (59), voltage-dependent K^+ channels (73), and insulin granule transport or priming (11,27).

MOLECULAR DETERMINANTS OF *Epac* ACTION

To appreciate the role of *Epac* in β-cell function, it is necessary to define the molecular basis for cAMPGEF signal transduction. The cAMPGEFs are cAMP-binding proteins first identified on the basis of their ability to stimulate the exchange of GDP for GTP at the guanyl nucleotide binding sites of the Rap1 and Rap2 small molecular weight G proteins (13,14). Two isoforms of cAMPGEFs exist and they are referred to as *Epac1* and *Epac2*—the *E*xchange *P*roteins directly *A*ctivated by *c*AMP (13). *Epac1* and *Epac2* are encoded by two distinct genes, and mRNAs corresponding to both isoforms of *Epac* are detectable in pancreas (14), islets (15), and insulin-secreting cell lines (15). The binding sites for cAMP located near the NH_2-terminus of cAMPGEFs bear structural resemblance to the B-type cAMP recognition site of

PKA regulatory subunit I (13,14). They contain the Pro-Arg-Ala-Ala motif that confers selectivity for binding of cAMP over cGMP. *Epac1* contains a single cAMP-binding domain, whereas *Epac2* contains two—a low affinity cAMP-binding domain designated as "A" and a higher affinity cAMP-binding domain designated as "B" (Fig. 2). The K_d for cAMP binding to full-length *Epac1* is 2.8 μmol/l (16). For *Epac2* the "A" and "B" binding sites exhibit a K_d of 87 and 1.2 μmol/l, respectively, when each binding site is studied in isolation (17). It may be concluded that cAMP exhibits a lower affinity for *Epac* than it does for PKA (K_d 0.1–1.0 μmol/l) (16). Such observations suggest that *Epac* is activated in a region of the cell where cAMP formation is strongly stimulated or in a subcellular compartment where the ability of cAMP to activate *Epac* is not inhibited by the activity of cAMP phosphodiesterase.

Analysis of the *Epac2* cDNA indicates that the two cAMP-binding domains are connected via a linker sequence to a GEF catalytic domain responsible for activation of Rap1. In the GEF domain are found three structurally conserved regions diagnostic of GEFs that interact with G proteins homologous to Ras. Binding of cAMP to cAMPGEFs activates Rap1 because it relieves an autoinhibitory function exerted by the cAMP-binding domain at the GEF's catalytic domain (18). Both isoforms of *Epac* contain a Ras exchange motif (REM) thought to be responsible for stabilization of the GEF. Also present is a DEP domain where homologies to *d*isheveled, *E*gl-10, and *p*leckstrin are found. The DEP domain may play a role in conferring to *Epac* its cell cycle–dependent targeting to intracellular membranes including the nuclear envelope (19). In this regard, a potential role for *Epac* in the regulation of mitosis is indicated because Cheng et al. (19) report that *Epac* localizes to the mitotic spindle and centrosomes during metaphase.

The interaction of *Epac* with Rap1 is of interest because this G protein is expressed in β-cells, where it is suggested to play a stimulatory role in glucose-dependent insulin secretion (20). Although the signal transduction properties of Rap1 are poorly understood, it is established that Rap1 acts as a molecular switch: it is active in its GTP-bound form and inactive in its GDP-bound form. Rap1 possesses an intrinsic GTPase activity so that the *Epac*-mediated activation of Rap1 is terminated by GTP hydrolysis. The hydrolysis of GTP is accelerated by *G*TPase *A*ctivating *P*roteins (GAPs), such as Rap1GAP, thereby completing a full cycle of activation and inactivation.

Exactly how activated Rap1 influences cellular function remains something of a mystery. Rap1 will antagonize

Epac2 Domain Structure
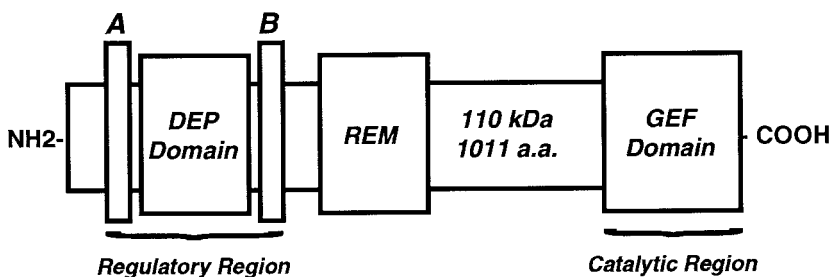
FIG. 2. Domain structure of *Epac2*. The regulatory region of *Epac2* contains the "A" and "B" cAMP-binding domains. This region of the molecule is responsible for inhibition of the catalytic GEF activity in the absence of cAMP. An increase of $[cAMP]_i$ relieves this autoinhibition. A DEP domain is responsible for association of *Epac2* with intracellular membranes, and a REM domain is responsible for stabilizing the tertiary structure of the GEF domain. The GEF domain catalyzes guanyl nucleotide exchange on Rap1, thereby activating the G protein. Illustration modeled after that of de Rooij et al. (17). Not drawn to scale.

TABLE 1
Signal transduction properties of *Epac*

Cellular function affected	Isoform of *Epac* activated	Immediate target of *Epac*	Reference
Insulin granule exocytosis	*Epac2*	Rim2, Piccolo	5,6,7
Primary β-cells, MIN6 cells			
Insulin granule priming	*Epac2*	SUR1, gSUR, CIC-3	11
Primary β-cells, MIN6 cells			
Ca^{2+}-induced Ca^{2+} release	*Epac2*	RYR-mediated	8,10
Primary β-cells, INS-1 cells			
ATP production	*Epac2*	CICR, Mitochondria	12
MIN6 cells			
Integrin-mediated cell adhesion	*Epac1*	Rap1	21
Ovarian Carcinoma Cells			
Renal H,K-ATPase	*Epac1*	Rap1, ERK1/2	75
Osteoblast proliferation	*Epac1, Epac2*	Rap1, B-Raf, ERK1/2	76
PCL-ε	*Epac1*	Rap2B,	23
HEK-293 cells			
c-Jun kinase (JNK)	*Epac1*	Unknown, Rap1-independent	26
HEK-293 cells			
Protein kinase B (PKB, *Akt*)	*Epac1*	Possibly Rap1-mediated	22
HEK-293 cells, thyroid cells			

Ras-mediated signal transduction, and a role for Rap1 in the integrin-mediated stimulation of cell adhesion is demonstrable (21). Furthermore, activation of Rap1 and/or Rap2 might play a role in the stimulation of protein kinase B (PKB, *Akt*) (22), phospholipase C-ε (PLC-ε) (23), and the ERK1/2 mitogen-activated protein kinases (MAPKs) (24). Interestingly, Rap1 has been reported to be located on secretory granule membranes, as expected if it plays a role in exocytosis (25). It should be noted that not all actions of *Epac* are necessarily Rap mediated. For example, *Epac* is reported to interact directly with the insulin granule–associated proteins Rim2 and Piccolo (6,7) and also the β-cell sulfonylurea receptor (SUR1) (5). *Epac* may also activate c-Jun kinase (JNK) independently of Rap1 (26). Table 1 summarizes signaling pathways that are proposed to be regulated by *Epac*. Note that because this is a new area of study, not all citations listed refer to β-cells.

NEW TOOLS FOR ANALYSIS OF *Epac* FUNCTION

Analysis of cAMPGEF signal transduction has been complicated by the lack of suitable tools with which to activate or inhibit *Epac* selectively. For example, the cAMP agonist Sp-cAMPS activates PKA and *Epac*, whereas the cAMP antagonist Rp-cAMPS is a poor blocker of *Epac* but an effective blocker of PKA (16). The limited ability of Rp-cAMPS to prevent activation of *Epac* may explain previous reports that cAMP exerts Rp-cAMPS–insensitive stimulatory effects on β-cell Ca^{2+} signaling and insulin secretion (27). With these points in mind, it is clear that previous studies demonstrating Rp-cAMPS–insensitive and apparently PKA-independent actions of GLP-1 must now be considered in a new light (28–30). It seems likely that at least some "PKA-independent" actions of GLP-1 may in fact be *Epac*-mediated.

An important new advance is the development of a cAMP analog (8-pCPT-2′-*O*-Me-cAMP) that activates *Epac* in a selective manner (31). The selectivity with which 8-pCPT-2′-*O*-Me-cAMP acts is explained by the fact that interactions of cAMP with PKA are contingent on the presence of a 2′-OH group on the ribose moiety of the cyclic nucleotide. Substitution of the 2′OH with a 2′-*O*-Me

group generates a cAMP analog exhibiting high affinity for *Epac* (K_d 2.2 μmol/l for *Epac1*) and a reduced affinity for PKA (K_d 20–30 μmol/l) (31). New studies demonstrate that in β-cells, 8-pCPT-2′-*O*-Me-cAMP increases $[Ca^{2+}]_i$ and stimulates exocytosis (10,11,32). These effects of 8-pCPT-2′-*O*-Me-cAMP are not blocked by the cAMP antagonist 8-Br-Rp-cAMPS or by the PKA inhibitors H-89 and KT5720 (10,11). Conversely, studies examining PKA-mediated signal transduction in the β-cell are likely to be facilitated by the use of N6-benzoyl-cAMP, a cAMP analog that preferentially activates PKA but not *Epac* (16).

It is also possible to evaluate the role of *Epac* in β-cell function by use of a "dominant negative" mutant *Epac2* in which inactivating G114E and G422D amino acid substitutions are introduced into the "A" and "B" cAMP-binding domains (6). Similarly, a dominant negative *Epac1* incorporating an R279E substitution within the single cAMP-binding domain has been described (19). Dominant negative *Epac2* blocks stimulatory actions of GLP-1 and 8-pCPT-2′-*O*-Me-cAMP on Ca^{2+} signaling and exocytosis in β-cells (6,8,10–12). It is also possible to generate constitutively active *Epac1* or *Epac2* by truncation of the cAMPGEF NH_2-terminus to remove autoinhibitory regulatory domains that bind cAMP (17). By use of transient transfection and overexpression of dominant negative or constitutively active *Epac*, efforts are now under way to evaluate the potential role of *Epac1* or *Epac2* in the regulation of β-cell stimulus-secretion coupling.

INTERACTIONS OF *Epac* WITH RIM2 AND PICCOLO

Seino and colleagues (5,6) first reported that *Epac2* interacts directly with the insulin granule–associated protein Rim2. This finding is of significance because the closely related isoform Rim1 is a *R*ab3-*i*nteracting *m*olecule that is demonstrated to play a central role in Ca^{2+}-dependent exocytosis. Studies of neurons indicate that Rim1 promotes the priming of synaptic vesicles, thereby rendering them release competent (33). Such findings are of significance because the studies of Sharp et al. (34) indicate that insulin granule priming is a necessary step for glucose-dependent first- and second-phase secretion. Therefore, by
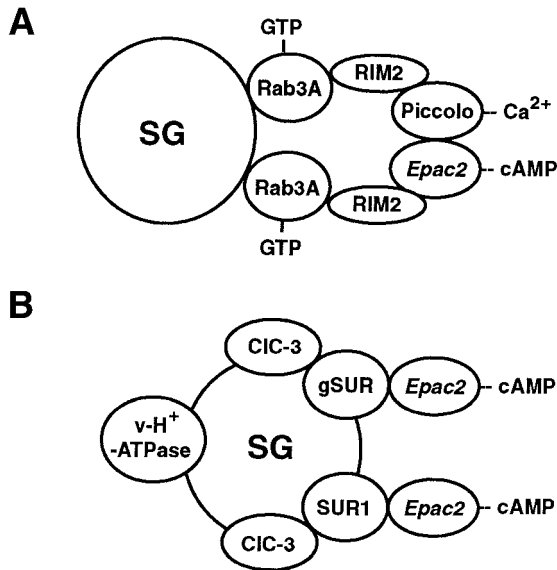
FIG. 3. *Epac* as a regulator of insulin granule exocytosis. *A:* Depicted are cAMP- and Ca²⁺-dependent interactions of *Epac2*, Rim2, and Piccolo based on an as yet to be established stoichiometry of 1:2:1. The *Epac2*-Rim2-Piccolo macromolecular complex interacts with the GTP-bound form of Rab3A to regulate the priming and exocytosis of secretory granules (SG) (5–7). *B: Epac2* stimulates exocytosis by interacting with insulin granule–associated sulfonylurea receptors (gSUR and SUR1) that regulate the activity of ClC-3 channels. Uptake of Cl⁻ into the granule facilitates granule acidification and priming mediated by the V-type H⁺-ATPase (11). Not illustrated are possible interactions of *Epac2* with plasma membrane–associated SUR1.

interacting directly with *Epac2*, Rim2 may accelerate insulin granule priming in a cAMP-regulated manner. The net effect is expected to be a facilitation of insulin secretion (Fig. 3*A*).

Epac2 is also reported to interact with Piccolo, a CAZ protein (*C*ytoskeletal matrix protein that associates with the *A*ctive *Z*one) originally described in studies of presynaptic nerve endings. Seino et al. (7) report that *Epac2*, Rim2, and Piccolo form a macromolecular complex, the stoichiometry of which has yet to be determined. Given that the binding of Ca²⁺ to Piccolo stimulates the formation of this complex, it may be speculated that the *Epac2*-Piccolo-Rim2 macromolecular complex may confer stimulatory effects of cAMP and Ca²⁺ on insulin secretion (Fig. 3*A*). This model of *Epac* signal transduction is consistent with the known ability of cAMP to facilitate Ca²⁺-dependent exocytosis in the β-cell (11,27).

The potential role of *Epac* as a regulator of insulin secretion is emphasized by studies demonstrating an interaction of Rim2 with the Ras-related G protein Rab3A (5). Rab3A is located on the cytoplasmic surface of insulin secretory granules, and it plays a role in the recruitment and docking of granules at the plasma membrane. Although the precise role of Rab3A in the regulation of insulin secretion has yet to be defined, Rab3A knockout (KO) mice exhibit a defect of insulin secretion, are glucose intolerant, and develop fasting hyperglycemia (35). However, unlike its role in the regulation of Rap1, *Epac2* does not appear to stimulate the exchange of guanyl nucleotides at Rab3A. Instead, *Epac2* appears to act downstream of Rab3A by virtue of its ability to form a complex with Rim2. It may be speculated that by interacting with Rim2, *Epac2* is targeted to docked secretory granules containing

Rab3A. Once situated at the docked secretory granules, *Epac2* might then mediate stimulatory effects of cAMP on exocytosis (Fig. 3*A*).

Mechanistically, a stimulatory effect of cAMP on insulin secretion might result from an *Epac*-mediated increase in the size of the readily releasable pool (RRP) of secretory granules. Rorsman et al. (8) propose that such an effect of *Epac2* is achieved by virtue of its ability to promote insulin granule acidification, a step necessary for granule priming. It is suggested that the binding of cAMP to *Epac2* promotes the opening of ClC-3 chloride channels that are located in the secretory granule membrane. cAMP-induced influx of Cl⁻ into the secretory granule lumen then creates an electromotive force that facilitates ATP-dependent H⁺ uptake mediated by a V-type H⁺-ATPase (Fig. 3*B*). How this takes place is not certain but it may involve sulfonylurea receptors expressed in the granule membrane (gSUR or SUR1) and/or plasma membrane (SUR1). These sulfonylurea receptors may act as transmembrane conductance regulators of ClC-3 channels so that the interaction of gSUR or SUR1 with ClC-3 might be *Epac* regulated. One attractive feature of this model is that it explains not only the ATP dependence of granule priming, but also the potential role of the *Epac2*-Rim2-Piccolo macromolecular complex as a determinant of the RRP size.

Epac FACILITATES FAST Ca²⁺-DEPENDENT EXOCYTOSIS

Epac appears to mediate stimulatory actions of GLP-1 on Ca²⁺-dependent insulin secretion. Evidence in support of this contention is provided by studies demonstrating that the action of GLP-1 is reduced by downregulation of the expression of *Epac2* in mouse islets (6). In these same studies, the action of GLP-1 is shown to be reproduced by 8-Br-cAMP, and the effect of 8-Br-cAMP is inhibited by overexpression of dominant negative *Epac2*. Stimuli that produce an increase of [Ca²⁺]ᵢ (e.g., KCl and carbachol) magnify the effect of 8-Br-cAMP, but this interaction is diminished under conditions of reduced *Epac2* expression. Such findings suggest that *Epac2* promotes Ca²⁺-dependent exocytosis, a conclusion supported by recent electrophysiological studies of mouse β-cells. Rorsman et al. (11) report that GLP-1 acts via *Epac2* to increase the size of the RRP of secretory granules by as much as 2.3-fold. This action of GLP-1 facilitates fast Ca²⁺-dependent exocytosis measured as an increase of membrane capacitance occurring within 200 ms of membrane depolarization. Facilitation of fast exocytosis is reduced by downregulation of *Epac2* expression or by transfection with dominant negative *Epac2*. It is also reproduced by 8-pCPT-2′-*O*-Me-cAMP in an Rp-cAMPS–insensitive manner, thereby confirming the likely involvement of *Epac2* in this signaling pathway (11).

PKA FACILITATES SLOW Ca²⁺-DEPENDENT EXOCYTOSIS

A substantial body of evidence indicates that *Epac* is unlikely to be the sole cAMP-binding protein subserving stimulatory effects of GLP-1 on insulin secretion. Overexpression of AKAP18 (an *A-K*inase *A*nchoring *P*rotein) in RINm5F cells facilitates insulin secretion measured in response to GLP-1 (36). This is significant because

AKAP18 interacts with the RII regulatory subunit of PKA and targets the kinase to the plasma membrane. In contrast, a sequestration of PKA and a concomitant inhibition of GLP-1–stimulated insulin secretion is observed after overexpression of a mutant AKAP18 that fails to target PKA to the plasma membrane (36). Inhibition of GLP-1–stimulated insulin secretion is also observed after exposure of islets to inhibitory peptides that disrupt the interaction of PKA with AKAPs (37). These findings demonstrate that under appropriate experimental conditions, the subcellular distribution of PKA dictates the effectiveness of GLP-1 as an insulin secretagogue. Recently, Wolf et al. (38) elaborated on these findings by providing evidence that it is the β isoform of the PKA catalytic subunit (Cβ) that mediates stimulatory actions of GLP-1 at the plasma membrane. Interestingly, GLP-1 is shown to stimulate the translocation of Cβ from the cytosol to the plasma membrane in betaTC6 insulin-secreting cells. Therefore, the subcellular distribution of activated catalytic subunit is not dictated solely by the distribution of AKAPs.

Seino et al. (3) report that treatment of mouse islets with *Epac2* antisense oligodeoxynucleotides reduces but does not abrogate the insulin secretagogue action of GLP-1. In contrast, exposure of mouse islets to the PKA inhibitor H-89 in the presence of *Epac2* antisense oligodeoxynucleotides results in a complete failure of GLP-1 to act. These findings are understandable in terms of electrophysiological studies of mouse β-cells demonstrating that cAMP acts via PKA to recruit secretory granules from a reserve pool to an RRP (11,27). This action of cAMP is responsible for a facilitation of slow Ca^{2+}-dependent exocytosis because it allows replenishment of the RRP under conditions in which a sustained increase of $[Ca^{2+}]_i$ occurs. Slow exocytosis is defined by Rorsman et al. (27) as an increase of membrane capacitance that occurs in response to repetitive membrane depolarization, but only after a delay of ~500 ms. The delay that precedes the appearance of slow exocytosis presumably reflects the time required to recruit reserve granules to the plasma membrane where they may then undergo docking and ATP-dependent priming in order to become release competent. The facilitation of slow exocytosis by cAMP is reproduced by GLP-1, is independent of *Epac*, and is blocked by Rp-cAMPS, thereby confirming the involvement of PKA. Importantly, the action of cAMP is not observed in the complete absence of Ca^{2+} (27). This key observation contrasts with one prior report that cAMP stimulates Ca^{2+}-independent exocytosis (39). However, the significance of Ca^{2+}-independent exocytosis is uncertain given that a Ca^{2+} channel blocker (nifedipine) abrogates stimulatory effects of GLP-1 on islet insulin secretion (30). Furthermore, Henquin et al. (40) report that under conditions of mild or stringent Ca^{2+} deprivation, GLP-1 does not potentiate glucose-dependent insulin secretion from mouse or rat islets.

"POST-PRIMING" ACTIONS OF PKA

One difficulty associated with the interpretation of prior patch clamp electrophysiological studies is that the measurements of exocytosis obtained in this manner do not necessarily reflect insulin secretion. This uncertainty results from the fact that β-cells contain not only large dense core secretory granules, but also synaptic vesicle-like structures that undergo Ca^{2+}-dependent exocytosis. Therefore, measurements of membrane capacitance might reflect vesicular rather than insulin granule exocytosis. If this were to be the case, it might be erroneously concluded that fast exocytosis plays a significant role in β-cell insulin secretion.

In an attempt to avoid these complications, Kasai and colleagues (41,42) measure exocytosis in mouse β-cells by means of carbon fiber amperometry. This method of analysis relies on the loading of β-cells with serotonin (5-HT), a monoamine that is selectively sequestered in large dense core secretory granules. By measuring the release of 5-HT, it is possible to detect exocytosis of single granules, thereby providing an indirect assay of insulin release. Amperometric determinations of exocytosis are not without interpretive difficulty because exposure of β-cells to 5-HT is known in some cases to interfere with glucose-dependent insulin secretion (43,44). However, using this approach, Kasai and colleagues (41,42) provide the surprising finding that PKA increases the release probability of docked secretory granules, an effect mediated at a step subsequent to ATP-dependent priming. This model of Kasai and colleagues is in contrast to that proposed by Rorsman and colleagues (11,27) because it suggests that fast Ca^{2+}-dependent exocytosis is regulated not only by *Epac*, but also by PKA.

One interesting aspect of the model proposed by Kasai and colleagues is that activation of PKA occurs in response to an elevation of $[ATP]_i$ secondary to glucose metabolism. It is proposed that the availability of ATP is a limiting factor for cAMP production and that adenylyl cyclase acts as a low-affinity ATP sensor, transducing a glucose-dependent increase of $[ATP]_i$ into an increase of $[cAMP]_i$ (41,42). In this model, the basal activity of adenylyl cyclase is suggested to be sufficiently high so as to allow significant cAMP production under conditions in which β-cells are exposed to glucose in the absence of cAMP-elevating agents such as forskolin. Kasai and colleagues then propose that glucose-dependent insulin secretion is contingent on the activation of PKA, which exerts a "postpriming" stimulatory effect at the docked insulin secretory granules. This model is in accordance with the role of ATP as a precursor for cAMP. It is also consistent with the ATP dependence of cAMP action because ATP serves as a phosphate donor in support of PKA-mediated phosphorylation.

What remains to be demonstrated is that glucose-dependent ATP production is a direct stimulus for cAMP biosynthesis. Although previous studies demonstrate that glucose increases the level of cAMP in the β-cell, this effect is usually attributed to Ca^{2+}-dependent activation of adenylyl cyclase (45). It is also uncertain whether activation of PKA is an absolutely necessary step in support of glucose-dependent insulin secretion. Kasai and colleagues. (41,42) report that Rp-cAMPS abrogates stimulatory effects of Ca^{2+} on exocytosis in mouse β-cells. On the basis of this observation, it is suggested that the postpriming action of PKA at insulin secretory granules is an essential determinant of stimulus-secretion coupling. However, prior studies directly measuring glucose-induced insulin secretion from intact islets offer no support for an absolute PKA dependence of secretagogue action (46,47). Further-

more, Kang et al. (9) demonstrate that under conditions in which PKA signaling is abrogated, exocytosis is readily observed in response to stimuli that mobilize Ca^{2+} from intracellular Ca^{2+} stores.

DEFECTIVE STIMULUS-SECRETION COUPLING IN SUR1-KO MICE

Whether it is *Epac* or PKA that plays a dominant role as a determinant of insulin secretion remains to be elucidated. In studies of mouse islets, Nakazaki et al. provide the surprising finding that PKA plays little or no role in the stimulation of insulin secretion by GLP-1 (30). Similarly, in studies of INS-1 insulin-secreting cells, inhibition of PKA by use of 8-Br-Rp-cAMPS fails to block exocytosis in response to the GLP-1 receptor agonist exendin-4 (9). In contrast, Gromada and colleagues provide clear evidence for a PKA-dependent mechanism by which GLP-1 stimulates insulin secretion in human and mouse β-cells (48–50). Perhaps one clue that resolves this quandary is provided by new studies examining the action of GLP-1 in SUR1 KO mice. When exposed to GLP-1, these mice secrete unusually small amounts of insulin despite the fact that GLP-1 remains effective as a stimulator of cAMP production (30,51). This loss of secretagogue action might be explained by the failure of β-cells to express ATP-sensitive K^+ channels (K_{ATP}) because SUR1 is a subunit of K_{ATP} channels and because prior studies demonstrate that K_{ATP} channels are inhibited by GLP-1 (29,48–50,52–54, but see ref. 55). Since the inhibition of K_{ATP} channels by GLP-1 is mediated at least in part by PKA (54), these studies of SUR1 KO mice hint at a predominant role for PKA as the principal effector by which GLP-1 acts.

Such a conclusion is tempered, however, by one recent study demonstrating that the signaling properties of *Epac* are downregulated in SUR1 KO mice (11). Although a facilitation of fast exocytosis by 8-CPT-2′-O-Me-cAMP is observed in wild-type mice, it is absent in the KO mice (11). To explain these findings, Eliasson et al. (11) propose that plasma membrane-associated SUR1 recruits *Epac2* to the inner cell surface where it mediates stimulatory effects of cAMP on secretory granule priming. Because SUR1 is also reported to be expressed in secretory granules (56) and because evidence exists for a sulfonylurea receptor–like protein (gSUR) in the granule membrane (11), the recruitment of *Epac2* might not be limited to the plasma membrane (Fig. 3*B*). If the model of Eliasson et al. should prove to be correct, it may be concluded that the stimulation of fast Ca^{2+}-dependent exocytosis by GLP-1 is an *Epac*-regulated event that might play a significant role in stimulus-secretion coupling.

Epac AS A DETERMINANT OF β-CELL Ca^{2+} SIGNALING

Recent studies of Kang and colleagues demonstrate that *Epac* is also likely to play a significant role in the regulation of β-cell Ca^{2+} signaling. Exposure of human β-cells or INS-1 insulin-secreting cells to the *Epac*-selective cAMP analog 8-pCPT-2-O-Me-cAMP produces a transient increase of $[Ca^{2+}]_i$ that reflects the mobilization of Ca^{2+} from intracellular Ca^{2+} stores (10). In both cell types this action of 8-pCPT-2-O-Me-cAMP is accompanied by exocytosis as measured by amperometric detection of released 5-HT (8,10). Two lines of evidence indicate that the

Ca^{2+}-mobilizing action of 8-pCPT-2-O-Me-cAMP is *Epac*-mediated. First, 8-pCPT-2-O-Me-cAMP remains effective under conditions in which INS-1 cells are treated with a cAMP antagonist (8-Br-Rp-cAMPS) or a selective inhibitor of PKA (H-89) (10). Second, overexpression of dominant negative *Epac2* blocks the action of 8-pCPT-2-O-Me-cAMP in transfected INS-1 cells (10). Interestingly, the Ca^{2+}-mobilizing action of 8-pCPT-2-O-Me-cAMP in INS-1 cells is not accompanied by a detectable increase of inositol trisphosphate (IP_3) production (10). This key finding demonstrates that the action of 8-pCPT-2-O-Me-cAMP in INS-1 cells is unlikely to be explained by *Epac*-mediated activation of PLC-ε with concomitant IP_3 production and the release of Ca^{2+} from IP_3 receptor (IP_3-R)-regulated Ca^{2+} stores.

Prior studies of β-cells offer competing hypotheses as to how cAMP mobilizes an intracellular source of Ca^{2+}. Liu et al. (57) and Tengholm et al. (58) report that cAMP promotes PKA-dependent sensitization of the IP_3-R, thereby facilitating CICR from the endoplasmic reticulum. Although this signaling mechanism may exist in β-cells, it is unlikely to explain the Ca^{2+}-mobilizing action of 8-pCPT-2-O-Me-cAMP. Instead, attention has recently focused on the possible involvement of an alternative source of intracellular Ca^{2+}, one that is regulated by ryanodine receptor (RYR) intracellular Ca^{2+} release channels. Kang et al. (10) report that pretreatment of human β-cells or INS-1 cells with ryanodine effectively blocks the increase of $[Ca^{2+}]_i$ measured in response to 8-pCPT-2-O-Me-cAMP. Because GLP-1 also stimulates ryanodine-sensitive CICR in β-cells (59–61), it may be speculated that a functional coupling exists between the GLP-1-R, *Epac*, and RYR (Fig. 4). Indeed, our most recent studies demonstrate that the GLP-1-R agonist exendin-4 stimulates CICR under conditions in which INS-1 cells are treated with 8-Br-Rp-cAMPS or H-89 (8). Furthermore, this action of exendin-4 is abrogated by overexpression of dominant negative *Epac2* (G.G.H. and G. Kang, unpublished observations).

What remains to be determined is exactly how activation of *Epac* is translated into the release of Ca^{2+} from Ca^{2+} stores. Such an effect might require direct interactions of *Epac* with Ca^{2+} release channels or, alternatively, *Epac* might act via an intermediary to promote kinase-mediated phosphorylation of the channels. Either mechanism might increase the sensitivity of Ca^{2+} release channels to cytosolic Ca^{2+}, thereby gating the channel from a closed to an open conformation (10). Such a mechanism of intracellular Ca^{2+} channel modulation might complement the previously reported ability of cAMP to sensitize Ca^{2+} release channels in a PKA-dependent manner (62–65).

IS MITOCHONDRIAL METABOLISM *Epac* REGULATED?

One promising avenue of future investigation concerns the possible importance of *Epac* as a determinant of mitochondrial metabolism. Recent studies of MIN6 insulin-secreting cells demonstrate that Ca^{2+} is a direct stimulus for glucose-dependent mitochondrial ATP production and that an increase of mitochondrial [ATP] is measurable under conditions in which CICR is triggered by cAMP (12). Although previous studies of β-cells offer competing interpretations as to how Ca^{2+} influences ATP production
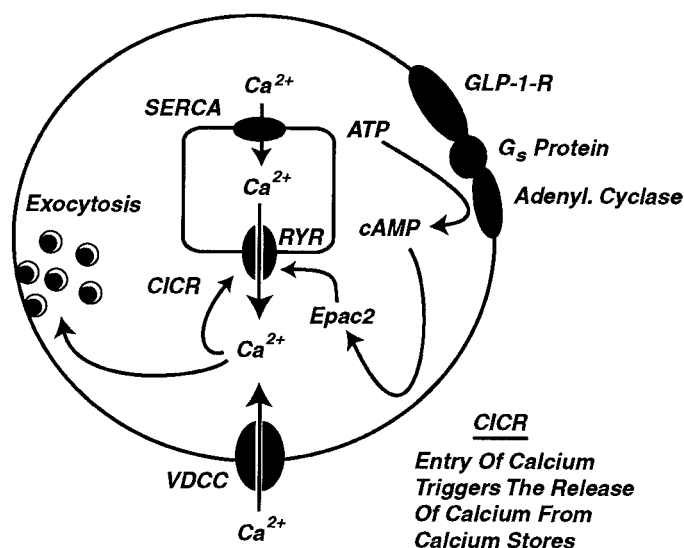
FIG. 4. The mobilization of intracellular Ca^{2+} by GLP-1 results from *Epac*-mediated sensitization of intracellular Ca^{2+} release channels. In this model the uptake of Ca^{2+} into the endoplasmic reticulum results from the activity of a sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA). The release of Ca^{2+} from the endoplasmic reticulum is initiated by the opening of RYR Ca^{2+} release channels in response to a simultaneous increase of cytosolic $[Ca^{2+}]$ and $[cAMP]$. This is a process of cAMP-regulated CICR. Under conditions in which β-cells are exposed to a stimulatory concentration of glucose, action potentials drive influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (VDCCs). The subsequent increase of $[Ca^{2+}]_i$ triggers CICR provided that RYR is sensitized by GLP-1. Sensitization of RYR may result from an ability of *Epac* to facilitate the action of Ca^{2+} at RYR. Note that exocytosis of secretory granules results from an increase of $[Ca^{2+}]_i$, which is a consequence of Ca^{2+} release and Ca^{2+} influx. Although not depicted, CICR may also originate in secretory granules where RYR is reportedly expressed (74).

(66,67), the findings obtained with MIN6 cells hint at a direct stimulatory effect of Ca^{2+} on mitchondrial dehydrogenases important to oxidative phosphorylation (Fig. 5).
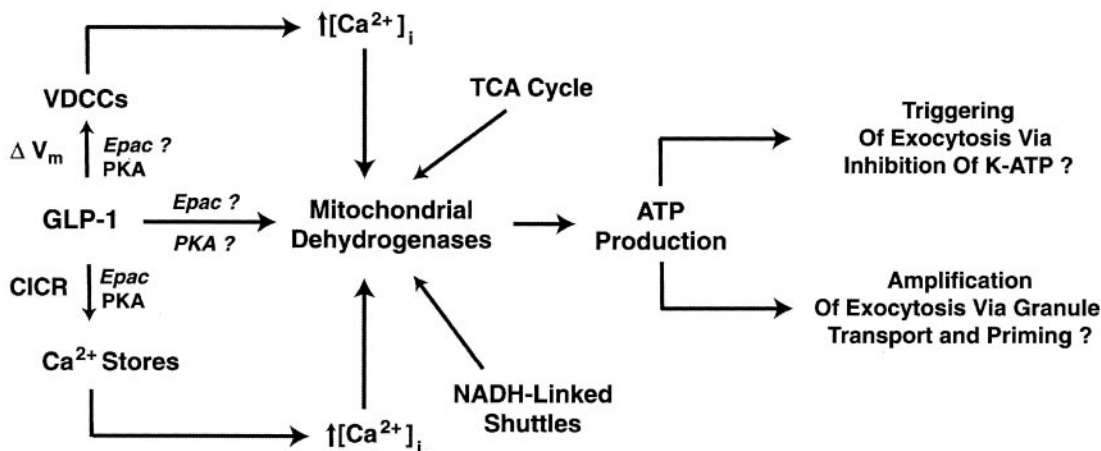
Indeed, Tsuboi et al. (12) report that GLP-1 acts via *Epac* to trigger CICR from ryanodine-sensitive Ca^{2+} stores, thereby raising the intramitochondrial concentrations of Ca^{2+} and ATP. These actions of GLP-1 are reproduced by forskolin, are inhibited by dominant negative *Epac2*, and are abrogated by pretreatment of MIN6 cells with a Ca^{2+} chelator (12).

Because GLP-1 stimulates free fatty acid production in β-cells (68), fatty acids might in theory serve as substrates for mitochondrial ATP production, thereby suggesting that the cAMP-dependent increase of mitochondrial [ATP] reported by Tsuboi et al. originates not only from glucose metabolism but also from lipid metabolism. It also remains to be determined to what extent GLP-1 acts via *Epac* to stimulate ATP production in authentic β-cells and, if so, whether the observed alterations of $[ATP]_i$ are translated into major alterations of K_{ATP} channel function, insulin granule transport, priming, and exocytosis (Fig. 5). If these criteria are met, the findings of Tsuboi et al. offer one simple explanation as to why all reported insulin secretagogue actions of GLP-1 are absolutely contingent on simultaneous exposure of β-cells to glucose.

CONCLUSION

This article focuses on the actions of GLP-1 that are likely to be *Epac* mediated and which might play a significant role in the regulation of β-cell stimulus-secretion coupling. Future studies will undoubtedly expand on this avenue of investigation to examine the possibility that *Epac* subserves stimulatory effects of glucagon, glucose-dependent insulinotropic peptide, or pituitary adenylyl cyclase activating polypeptide on β-cell function. Moreover, it will be of interest to ascertain whether such effects complement the previously reported actions of GLP-1 to produce long-term alterations in the pattern of gene expression

FIG. 5. GLP-1 stimulates glucose-dependent mitochondrial ATP production by initiating an increase of $[Ca^{2+}]_i$. In this model, the dual actions of GLP-1 to promote depolarization-induced Ca^{2+} influx and to release Ca^{2+} from intracellular stores result in an increase of intramitochondrial $[Ca^{2+}]$ and a stimulation of Ca^{2+}-sensitive mitochondrial dehydrogenases (12). These dehydrogenases are components of the TCA cycle and NADH-linked shuttles. Modest changes of cytosolic $[Ca^{2+}]$, as do occur in response to CICR, produce a large increase of intramitochondrial $[Ca^{2+}]$ due to the activity of the mitochondrial uniporter Ca^{2+} transport mechanism. Therefore, the concentration of Ca^{2+} within the mitochondria reaches a level commensurate with the affinity of these dehydrogenases for Ca^{2+}. Note that depolarization-induced Ca^{2+} influx in response to GLP-1 results from an inhibitory action of the hormone at K_{ATP} channels (52). This effect is PKA mediated (54) and may also result from direct interactions of *Epac* with SUR1 (5). The release of Ca^{2+} from intracellular Ca^{2+} stores results from stimulatory actions of GLP-1 at RYRs and IP$_3$-Rs. Both types of Ca^{2+} release channels are regulated by PKA (57,62), whereas current evidence suggests a selective action of *Epac* at RYR (8,10). It has yet to be determined if the increase of $[ATP]_i$ measured in response to GLP-1 is of sufficient magnitude to result in the inhibition of K_{ATP} channels, thereby facilitating the triggering pathway of glucose-dependent insulin secretion. It is also not certain if ATP generated in this manner exerts a direct effect at the insulin secretory granules, thereby facilitating granule transport, priming, and exocytosis via the amplification pathway of insulin secretion.

important to β-cell neogenesis, differentiation, and survival (69–71). Should this prove to be the case, interest within the diabetes research community might spur efforts to develop new pharmacological agents that activate *Epac* in a direct and selective manner.

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Shibasaki et al. (Shibasaki T, Sunaga Y, Fujimoto K, Kashima Y, Seino S: Interaction of ATP sensor, cAMP sensor, Ca^{2+} sensor, and voltage-dependent calcium channel in insulin granule exocytosis (*J Biol Chem*. In press) have recently provided additional biochemical evidence for a direct protein-protein interaction between *Epac2* and the nucleotide binding fold-1 (NBF-1) of SUR1.

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