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REVIEW

Cell signalling in insulin secretion: the molecular targets of ATP, cAMP and sulfonylurea

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Abstract Clarification of the molecular mechanisms of insulin secretion is crucial for understanding the pathogenesis and pathophysiology of diabetes and for development of novel therapeutic strategies for the disease. Insulin secretion is regulated by various intracellular signals generated by nutrients and hormonal and neural inputs. In addition, a variety of glucose-lowering drugs including sulfonylureas, glinide-derivatives, and incretin-related drugs such as dipeptidyl peptidase IV (DPP-4) inhibitors and glucagon-like peptide 1 (GLP-1) receptor agonists are used for glycaemic control by targeting beta cell signalling for improved insulin secretion. There has been a remarkable increase in our understanding of the basis of beta cell signalling over the past two decades following the application of molecular biology, gene technology, electrophysiology and bioimaging to beta cell research. This review discusses cell signalling in insulin secretion, focusing on the molecular targets of ATP, cAMP and sulfonylurea, an essential metabolic signal in glucose-induced insulin secretion (GIIS), a critical signal in the potentiation of GIIS, and the commonly used glucose-lowering drug, respectively.

Keywords ATP · cAMP · Epac · Incretin · Insulin secretion · K_{ATP} channel · Review · Sulfonylurea

Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
DAG	Diacylglycerol
DPP-4	Dipeptidyl peptidase IV
ECFP	Enhanced cyan fluorescent protein
Epac	Exchange protein activated by cAMP
Exoc3l	Exocyst complex component 3-like
EYFP	Enhanced yellow fluorescent protein
FRET	Fluorescence resonance energy transfer
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GIIS	Glucose-induced insulin secretion
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GR	Glucose-responsive
IP_3	Inositol 1,4,5-trisphosphate
K_{ATP} channel	ATP-sensitive K^+ channel
PHHI	Persistent hyperinsulinaemic hypoglycaemia of infancy
PKA	Protein kinase A
RP	Reserve pool
RRP	Readily releasable pool
SUR	Sulfonylurea receptor
TIRFM	Total internal reflection fluorescence microscopy
TM	Transmembrane
TRP	Transient receptor potential
VMH	Ventromedial hypothalamus

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Introduction

The blood glucose level is tightly controlled by insulin secretion from pancreatic beta cells and insulin action in target tissues such as liver, muscle and adipose tissue. Pancreatic beta cells secrete an appropriate amount of insulin in a process that is precisely regulated temporally to maintain glucose homeostasis. Insulin secretion is regulated by various factors, including nutrients and hormonal and neural inputs to the beta cells, among which glucose is the most important physiological regulator. Beta cell dysfunction impairs normal regulation of insulin secretion and leads to diabetes or hypoglycaemia. The mechanisms of insulin secretion have been studied extensively both in vivo and in vitro in the 50 years since the establishment of the radioimmunoassay for insulin [1]. Our understanding of the mechanisms of insulin secretion was deepened but remained incomplete. By the early 1980s, the major intracellular signals in pancreatic beta cells for insulin secretion had been identified by pharmacological, physiological and biochemical methods. These include Ca^{2+}, ATP, cAMP and phospholipid-derived molecules such as diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [2–4].

Glucose-induced insulin secretion (GIIS) is the principal mechanism of insulin secretion (Fig. 1a). Early studies proposed two models of beta cell glucoreceptor signalling in GIIS: the regulatory-site model [5] and the substrate-site model [6, 7], although most studies supported the latter model [8]. Following the discovery of the ATP-sensitive K^+ (K_{ATP}) channel in cardiomyocytes by electrophysiology [9], the K_{ATP} channel and the glucose-regulated K^+ channel were also found in pancreatic beta cells [10–12]. As the K_{ATP} channel couples glucose metabolism to electrical activity of the beta cell [13], the discovery of the channel supported the notion that glucose metabolism is essential for GIIS. Accumulating evidence indicates that glucose induces insulin secretion by two different pathways: the triggering pathway (K_{ATP} channel-dependent pathway) and the metabolic amplifying pathway (K_{ATP} channel-independent pathway), the former of which is essential for GIIS [14]. According to the current consensus on the triggering pathway of GIIS, glucose transported into the beta cell through glucose transporters is rapidly metabolised to yield an increase in the ATP concentration (ATP/ADP ratio), which causes closure of K_{ATP} channels and depolarisation of the cell membrane. Membrane depolarisation opens the voltage-dependent Ca^{2+} channels, allowing Ca^{2+} influx. The resultant rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the beta cell leads to fusion of insulin granules to the plasma membrane in a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent process. In contrast to this well-established triggering pathway, the metabolic amplifying pathway is more complex and its mechanism less well understood. It has been suggested that metabolic signals generated by glucose, such as ATP, act on steps in the secretory process distal to the $[Ca^{2+}]_i$ rise [15–17]. However, this pathway does not influence insulin secretion if $[Ca^{2+}]_i$ is not increased; the metabolic amplifying pathway does not function if the triggering pathway is not operational. In addition to metabolic signals generated by glucose, intracellular signals such as cAMP, DAG and IP_3, evoked by hormonal and neuronal inputs, are important for normal regulation of insulin secretion (neurohormonal amplifying pathway) [14]. Lipid metabolism is also important for regulating as well as modulating insulin secretion [18]. The molecular bases for stimulus-secretion coupling in GIIS and its potentiation were largely unknown until the early 1990s. By utilising molecular biology, gene technology and bioimaging, many regulators and targets of intracellular signals in insulin secretion have been identified, greatly enhancing our understanding of GIIS. This review discusses the targets of ATP, cAMP and the glucose-lowering drug sulfonylurea and their roles in GIIS, based on our recent studies.

Dynamics of insulin secretion

Insulin secretion is a highly dynamic process. Glucose induces insulin secretion in a biphasic pattern: there is an initial component (first phase) that develops rapidly but lasts only a few minutes, and this is followed by a progressively increasing or sustained component (second phase) [14, 19, 20]. Loss of first phase secretion and reduced second phase secretion are characteristic features of type 2 diabetes. It is known that there is a decrease in the first phase of GIIS in the early stage of type 2 diabetes and in impaired glucose tolerance [21].

By analogy with the exocytosis of neurotransmitters in neurons [22], insulin granule exocytosis is thought to involve several steps, including recruitment, docking, priming and fusion [23]. It has been suggested that secretory vesicles in pancreatic beta cells exist in functionally distinct pools and that the sequential release of these pools underlies the separable components in the dynamics of exocytosis [20]. Pancreatic beta cells contain at least two pools of insulin secretory granules that differ in release competence: a reserve pool (RP) accounting for the vast majority of granules, and a readily releasable pool (RRP) accounting for the remaining <5%. A current hypothesis maintains that the first phase of GIIS is caused by release of RRP granules and that the second phase of GIIS represents a subsequent supply of new granules mobilised from the RP [14, 20, 24].

Investigation of insulin granule dynamics has recently been refined by use of the total internal reflection fluorescence microscopy (TIRFM) system [25–28]. TIRF is a technology that provides a means of selectively exciting fluorophores in an aqueous or cellular environment very near a solid surface (within 100 nm) without exciting fluorescence from regions

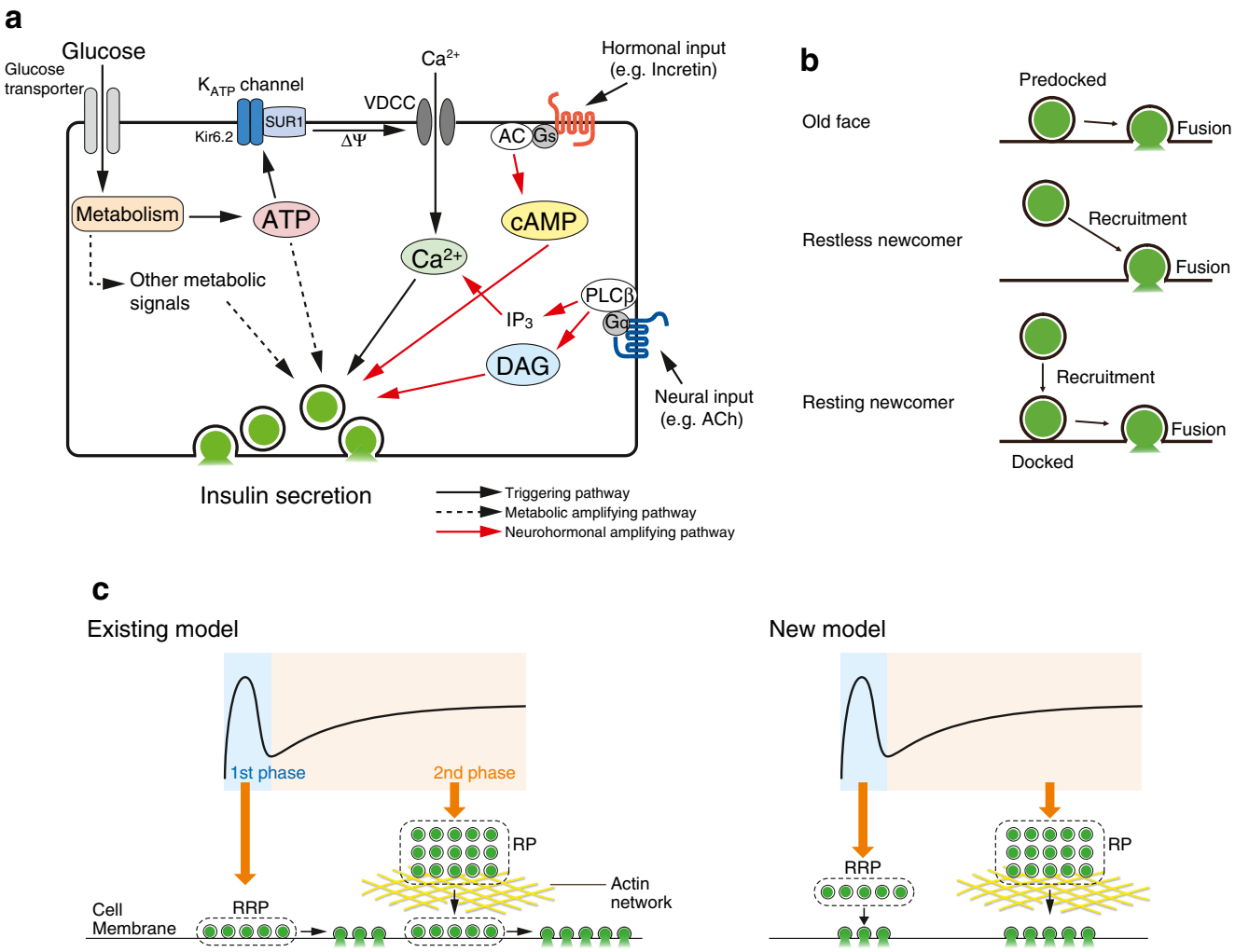


Fig. 1 **a** Glucose-induced insulin secretion and its potentiation. Glucose is transported thorough the glucose transporter into the pancreatic beta cell. Metabolism of glucose increases ATP production (ATP/ADP ratio), closing the K_{ATP} channels, depolarising beta cell membrane ($\Delta\Psi$), opening the voltage-dependent Ca^{2+} channels (VDCCs) and allowing Ca^{2+} influx, thereby triggering insulin secretion (formerly called the K_{ATP} channel-dependent pathway, presently called the triggering pathway). In addition to the triggering pathway, metabolic signals generated by glucose metabolism amplify insulin secretion (formerly called the K_{ATP} channel-independent pathway, presently called the metabolic amplifying pathway). Insulin secretion is also amplified by hormones and neurotransmitters that generate intracellular signals such as cAMP, DAG, and IP_3 (neurohormonal amplifying pathways). PLCβ, phospholipase C-β; AC, adenylyl cyclase; ACh, acetylcholine. **b** Modes of insulin granule exocytosis. There are three modes of insulin granule exocytosis based on the dynamics of the granules. 'Old face': predocked granules that are fused to the plasma membrane by stimulation. 'Restless newcomer': granules that are newly recruited by stimulation and immediately fused to the plasma membrane. 'Resting newcomer': granules that are newly recruited by stimulation, docked and fused to the plasma membrane by stimulation. Modified from [27] with permission. Copyright 2007 National Academy of Sciences, U.S.A. **c** Models of glucose-induced insulin secretion. In the existing model of GIIS, the first phase of insulin secretion results from an RRP comprising predocked insulin granules (old face); the second phase secretion results from an RP comprising granules located farther away (resting newcomer), granules that are newly recruited upon stimulation, docked, and fused to the plasma membrane. In the new model, both phases are caused by restless newcomer granules that are recruited upon stimulation and immediately fused to the plasma membrane without docking. Modified with permission of the American Society for Clinical Investigation, from [31]; permission conveyed through Copyright Clearance Center, Inc.

further from the surface [29]. This unique feature of TIRFM analysis has has led to its application in various different areas of biochemistry and cell biology. A previous TIRFM study reported that insulin granule exocytosis occurs in two modes [26]. In one mode (mode 1), fusion events are caused by granules that are predocked to the plasma membrane (referred to as 'previously docked granules' in [26] and 'old face' in [27]). In the other mode, fusion events are caused by granules that are newly recruited to the plasma membrane ('newcomer'). Detailed analyses of insulin granule dynamics induced by various stimuli using primary cultured pancreatic beta cells show that 'newcomer' can be classified into two modes: one mode (mode 2), in which granules are newly recruited and immediately fused to the plasma membrane without docking

(a docking state that can barely be detected by TIRFM) ('restless newcomer'), and another mode (mode 3) in which granules are newly recruited, docked and then fused to the plasma membrane ('resting newcomer') [27] (Fig. 1b). The three modes of insulin granule exocytosis have been confirmed by other studies [28, 30]. Unlike the original model of GIIS, in which the first phase results from the RRP comprising predocked granules and the second phase from RP, a new model in which both phases of GIIS are caused by 'restless newcomer' has been proposed (Fig. 1c) [31].

In contrast, most K^+-induced insulin granule exocytosis that occurs immediately and transiently after stimulation represents the release of predocked granules ('old face') [27, 32]. The dynamics of insulin granule exocytosis vary according to whether stimulation is due to K^+ or glucose. As K^+ stimulation elicits only Ca^{2+} influx and glucose stimulation generates various metabolic signals such as ATP in addition to Ca^{2+} influx in pancreatic beta cells, this difference in intracellular signal may underlie the distinct modes of exocytosis.

Various proteins associated with insulin granule exocytosis have been identified [23, 33], among which Rab-interacting molecule 2 (Rim2, Rim2α) was identified as a molecule interacting with exchange protein activated by cAMP (Epac) 2A (cAMP-GEFII) [34]. In addition to Epac2A, Rim2α interacts with various exocytosis-related molecules, at least in vitro, including Rab3 [34], Munc13-1 [35], Rab8 [36], ELKS [37, 38], Piccolo [39], and synaptotagmin 1 [40]. Although synaptotagmin 1 is produced in insulinoma cells, the synaptotagmin genes expressed in primary mouse beta cells are those encoding synaptotagmin 7 [41] and 9 [42]. Rim2α null (*Rim2α*$^{-/-}$) mice exhibit a marked impairment in glucose tolerance [43]. Analysis by TIRFM shows that both K^+-induced insulin granule exocytosis and glucose-induced insulin granule exocytosis, especially the first phase, are severely impaired in pancreatic beta cells of Rim2α null mice [43]. Rim2α has been found to determine the docking and priming states depending on interaction with Rab3 or Munc13-1, respectively.

The exocyst is an octameric protein complex that ensures spatial docking or tethering of exocytotic vesicles to fusion sites of the plasma membrane [44]. Eight subunits of the exocyst complex are expressed in both pancreatic islets and MIN6 cells. Exocyst complex component 3-like (Exoc3l), an isoform of Sec6, the core subunit of the exocyst complex, was identified by in silico screening [45]. Exoc3l forms tertiary complexes consisting of Sec5, Sec8 and Sec10, all of which are binding partners of Sec6. Exoc3l is suggested to be involved in the regulated exocytosis of insulin granules through formation of the exocyst complex.

cAMP-increasing ligands potentiate both the first phase and second phase of GIIS [4]. However, the potentiating effect of cAMP occurs only at glucose concentrations above a certain threshold [46]. cAMP also affects various steps of insulin secretion. In normal pancreatic islets, the in vitro concentration dependence of GIIS displays a sigmoidal curve [47], in which a glucose concentration exceeding 6 mmol/l is required to trigger insulin secretion. In addition, it has been reported that glucagon-like peptide 1 (GLP-1) renders glucose-insensitive beta cells glucose-competent, probably by modulating K_{ATP} channel activity [48]. These findings suggest a mechanism by which cAMP might induce beta cell glucose responsiveness. Using a pancreatic perfusion system, we recently found that pretreatment with GLP-1 or glucose-dependent insulinotropic polypeptide (GIP) improved glucose responsiveness to some extent in Kir6.2 null (*Kir6.2*$^{-/-}$) mice, in which almost no insulin secretion in response to glucose is detected [49], but the effect of GLP-1 was stronger. The dynamics of GIIS is usually assessed by the insulin secretory response to a large and prompt change in glucose concentration, e.g. from 2.8 to 16.7 mmol/l in 1 min, using perfusion of the pancreas and perifusion of pancreatic islets. However, such a drastic change in glucose concentration is unlikely to occur in the physiological state. As regards the perfusion of the pancreases of wild-type mice, when the glucose concentration was increased in a stepwise manner (1.4 mmol/l every 5 min) from 2.8 to 12.5 mmol/l in the absence of cAMP-increasing agents (8-bromo-cAMP or GLP-1), no insulin secretion was evoked (Fig. 2a) [50]. Interestingly, the presence of these agents resulted in a dramatic induction of GIIS (Fig. 2a, b). Similar results also were found in Kir6.2 null mice. This GIIS was almost completely abolished by treatment with niflumic acid, indicating that cAMP signalling also evokes glucose responsiveness by activating niflumic acid-sensitive channels (Fig. 2c). Niflumic acid is often used to block Cl^- channels but it also acts on other channels, including transient receptor potential (TRP) channels. Since removal of Na^+ did not abolish the membrane depolarisation caused by glucose and cAMP [50], Na^+ influx through TRP channels is unlikely to be a contributor to initial membrane depolarisation; Ca^{2+}-activated Cl^- channels are the more likely candidate for the niflumic acid-sensitive channels responsible for depolarisation caused by glucose and cAMP. These findings indicate that cAMP signalling is important not only for potentiation of GIIS but also for induction of glucose responsiveness in insulin secretion.

The role of cAMP signalling in insulin granule exocytosis has also been investigated by TIRFM. The cAMP analogue 8-bromo-cAMP alone did not cause either significant docking or fusion events of insulin granules. However, 8-bromo-cAMP clearly enhanced the frequency of glucose-induced fusion events in both the first phase and the second phase. 8-bromo-cAMP promoted fusion events by increasing only 'restless newcomer'. Comparison of the fusion sites induced by glucose stimulation and those induced by 8-bromo-cAMP stimulation showed that new fusion sites appeared upon 8-bromo-cAMP stimulation, suggesting that

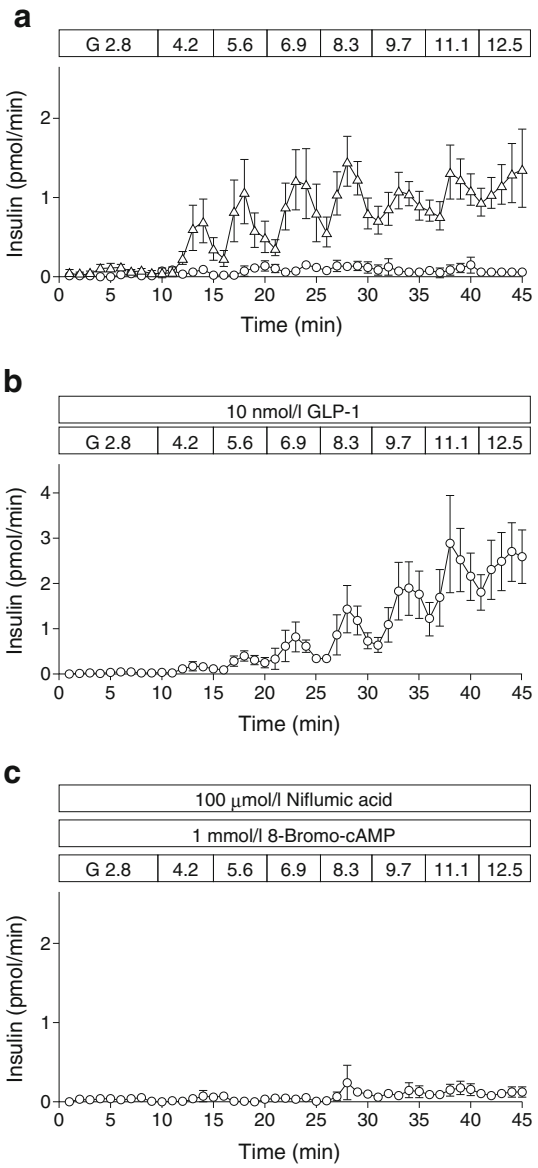


Fig. 2 **a** Insulin secretion in response to small and stepwise increases (increment of 1.4 mmol/l per 5 min) in glucose concentration in the absence (white circles) and presence (white triangles) of 1 mmol/l 8-bromo-cAMP. **b** Insulin secretion in response to small and stepwise increases in glucose concentration in the absence of 10 nmol/l GLP-1. **c** Effect of niflumic (100 μmol/l) acid on insulin secretion in response to small and stepwise increases in glucose concentration in the presence of 1 mmol/l 8-bromo-cAMP. Reproduced from [50] with permission of Springer Science+Business Media

cAMP signaling also participates in the spatial regulation of insulin granule exocytosis [27].

The K_{ATP} channel as a target of ATP and sulfonylurea

The K_{ATP} channel links cellular metabolic status to the electrical activity of pancreatic beta cells [10–12] and is a key molecule in the regulation of GIIS. The beta cell K_{ATP} channel was also suggested to be the target for sulfonylurea [51], a widely used drug in the treatment of type 2 diabetes. However, whether the target (receptor) for sulfonylurea was the K_{ATP} channel itself or a molecule associated closely with the K_{ATP} channel was not known. In 1995, a receptor (SUR1) for sulfonylurea was cloned by Aguilar-Bryan and colleagues [52] (Fig. 3a). SUR1 was found to be a member

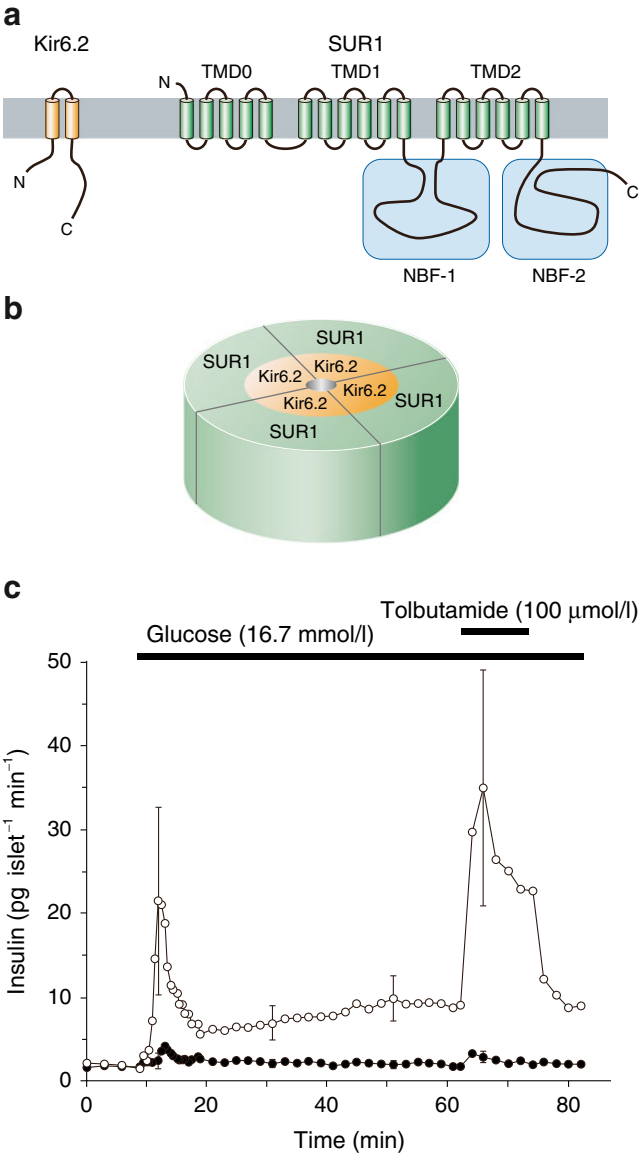


Fig. 3 **a** Kir6.2 and SUR1. Kir6.2, which belongs to the inwardly rectifying K^+ channel family, is the pore-forming subunit. SUR1, which belongs to the ATP-binding cassette transporter family, is the regulatory subunit. **b** Subunit structure of the beta cell K_{ATP} channel. A beta cell K_{ATP} channel is composed of Kir6.2 and SUR1 with 4 to 4 stoichiometry. **c** Insulin secretion from perifused pancreatic islets of Kir6.2 null mice (black circles) and wild-type mice (white circles). NBF, nucleotide binding fold; TMD, transmembrane domain. Reproduced from [62] with permission. Copyright 1998 National Academy of Sciences, USA

of the ATP-binding cassette (ABC) transporter family [52]. However, expression of SUR1 alone in *Xenopus oocytes* did not generate K^+ currents, demonstrating that SUR1 is not itself the K_{ATP} channel. At almost the same time that SUR1 was cloned, we identified a novel member of the inwardly rectifying K^+ channel family, designated BIR (now known as Kir6.2), by homology screening of the insulin-secreting cell line MIN6 cDNA library using uKATP-1 (now Kir6.1) as a probe (Fig. 3a) [53]. Like SUR1, expression of Kir6.2 alone in mammalian cells did not produce K^+ channel activity. We therefore explored the possibility that Kir6.2 generates K^+ channel activity by coupling with SUR1. Coexpression of Kir6.2 and SUR1 in mammalian cells generated ATP-sensitive and sulfonylurea-sensitive K^+ currents with electrophysiological and pharmacological properties similar to those of beta cell K_{ATP} channels [53], demonstrating that the beta cell K_{ATP} channel is composed of Kir6.2, a pore-forming subunit, and SUR1, a regulatory subunit (Fig. 3a). Reconstitution of the beta cell K_{ATP} channel currents from Kir6.2 and SUR1 was also reported using a *Xenopus oocyte* expression system by Ashcroft and her colleagues [54]. The beta cell K_{ATP} channel functions as a hetero-octameric complex comprising a tetramer of the Kir6.2 subunit and a tetramer of the SUR1 subunit (Fig. 3b). SUR2 (SUR2A and SUR2B) was subsequently cloned. It is now known that differing combinations of Kir6.2 or Kir6.1 and SUR1, SUR2A or SUR2B constitute different K_{ATP} channels with distinct nucleotide sensitivities and pharmacological properties in various cell types [55, 56], where they function as metabolic sensors [57, 58]. A two-site (A site and B site) model for the interaction of SUs and glinides with SUR has been proposed [59, 60]. The A site is located on the eighth cytosolic loop (between transmembrane segments [TM] 15 and 16), which is specific for SUR1, and the B site is located on the third cytosolic loop (between TM 5 and 6), which is very similar in all SURs. Based on this model, SUs and glinides can be divided into three groups. The first group (which includes tolbutamide, gliclazide and nateglinide) binds specifically to the A site of SUR1; the second group (which includes glibenclamide, known as glyburide in the USA and Canada, and glimepiride) binds to the B sites of both SUR1 and SUR2A as well as to the A site of SUR1; the third group (which includes meglitinide and repaglinide) binds to the B site of SUR1 and SUR2A.

Studies of various K_{ATP} genetically engineered mice [61–64] clearly show the essential role of the K_{ATP} channel for GIIS and sulfonylurea-induced insulin secretion (Fig. 3c). Under certain conditions in Kir6.2 null mice [63, 65], GIIS was detected to some extent, indicating that the metabolic amplifying pathway contributes at least in part to GIIS. However, during stimulation with glucose alone, the metabolic amplifying pathway is ineffective as long as the triggering pathway is inoperative. The triggering and metabolic amplifying pathways in GIIS have recently been reviewed in detail [14]. Mutations of the beta cell K_{ATP} channels cause neonatal diabetes or persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI), depending on gain-of function (activating) mutation or loss-of-function mutation, respectively. The pathophysiology of PHHI and neonatal diabetes owing to mutations of Kir6.2 and SUR1 have been discussed in detail in recent review articles [66–68].

The ventromedial hypothalamus (VMH) has been shown to possess the highest density of glucose-responsive (GR) neurons, which play a critical role in glucose homeostasis and are involved in glucagon secretion during hypoglycaemia [69]. The VMH K_{ATP} channel has been found to consist of Kir6.2 and SUR1 [70], which is identical to the beta cell K_{ATP} channel. Recovery from systemic hypoglycaemia induced by insulin injection was severely impaired in Kir6.2 null mice due to a marked reduction in glucagon secretion in vivo in these mice. Glucagon secretion in response to low glucose concentrations in isolated pancreatic islets from Kir6.2 null mice to that islets from wild-type mice, indicating a normal response of glucagon secretion from alpha cells [71]. Administration of 2-deoxyglucose (2DG) into the intracerebroventricle, which is known to induce neuroglucopenia in the hypothalamus and to stimulate glucagon secretion through activation of autonomic neurons, produced an increase in glucagon secretion in normal mice but not in Kir6.2 null mice. Thus, the K_{ATP} channels in the VMH function as glucose sensors for glucagon secretion during hypoglycemia. Beta-cell and VMH K_{ATP} channels act in concert as peripheral and central glucose sensors in the maintenance of glucose homeostasis.

It was suggested that some sulfonylureas enhance glucose uptake in skeletal muscles [72, 73]. The glucose-lowering effect by insulin injection at a relatively low dose is significantly increased in Kir6.2 null mice compared with that in normal mice, suggesting that insulin sensitivity is enhanced in Kir6.2 null mice [62]. In fact, glucose uptake in some skeletal muscles is increased in Kir6.2 null mice [74]. The involvement of the K_{ATP} channels in glucose uptake in skeletal muscles also is indicated by a study of SUR2 null ($SUR2^{-/-}$) mice [75]. Thus, closure of the K_{ATP} channels in skeletal muscles can enhance glucose transport. K_{ATP} channels in pancreatic beta cells, hypothalamus and skeletal muscle are critically involved in the maintenance of glucose homeostasis [76].

Epac2A (cAMP-GEFII) as a target of cAMP

Since the discovery of cAMP as an intracellular second messenger, it has been shown to mediate a variety of cellular responses. Various hormones and neurotransmitters, including GLP-1 [77–79], GIP [77, 79], vasoactive intestinal polypeptide (VIP) [80] and pituitary adenylate cyclase-

activating polypeptide (PACAP) [80], potentiate insulin secretion by promoting cAMP generation in pancreatic beta cells. Eight adenylyl cyclase isoforms (types I–VIII) are expressed in pancreatic islets and beta cell lines [81, 82]. In fact, MDL12330A, an adenylyl cyclase inhibitor, completely blocks both GLP-1- and GIP-induced cAMP production in islets and also markedly reduces both GLP-1- and GIP-potentiated insulin secretions [83].

Until recently, the action of cAMP in insulin secretion was thought to primarily be mediated by protein kinase A (PKA), which phosphorylates various proteins associated with the secretory process [84]. Kir6.2, the pore-forming subunit of K_{ATP} channels, and the α-subunit of the voltage-dependent Ca^{2+} channel can be phosphorylated by PKA on stimulation in beta cell lines [85, 86]. GLUT2 can also be phosphorylated by GLP-1 in purified beta bcells [87]. Although phosphorylation of these proteins influences their activities [85–87], a direct effect of phosphorylation on insulin secretion has not been established. We recently found that in MIN6 cells, Rip11, an effector of the small G-protein Rab11, participates in the potentiation of exocytosis by cAMP plus glucose stimulation but not in that of glucose stimulation alone [88]. In addition, Rip11 was found to be phosphorylated by PKA in MIN6 cells. These findings indicate that Rip11, as a substrate of PKA, is involved in the regulation of insulin secretion potentiated by cAMP in pancreatic beta cells.

It is now known that cAMP also potentiates GIIS by a PKA-independent mechanism [84] (Fig. 4a). This mechanism is mediated by the cAMP-binding protein Epac2 (also referred to as cAMP-GEFII; Fig. 4b). Two Epacs, Epac1 (cAMP-GEFI) and Epac2, have been identified [89–91]. Both Epac proteins possess guanine nucleotide exchange factor (GEF) activity towards the small G-proteins Rap1 and Rap2 in a cAMP-dependent manner [89–91]. It has become apparent that Epac proteins are cAMP sensors that regulate several cellular processes independently of PKA. *Epac1* mRNA is ubiquitously expressed, while *Epac2* mRNA is mainly expressed in neurons, neuroendocrine cells and endocrine cells [34, 89, 90]. Epac1 and Epac2 are each encoded by two genes. Structurally, both Epac proteins have common features: an amino-terminal regulatory region harbouring cAMP-binding domains and a Dishevelled, Egl-10, and Pleckstrin (DEP) domain and a carboxyl-terminal catalytic region harboring an Ras exchange motif (REM) domain, an Ras-association (RA) domain, and a CDC25-homology domain for GEF activity. However, Epac1 possesses one cAMP-binding domain, while Epac2 possesses two cAMP-binding domains. X-ray crystallographic analysis of the inactive and active forms of Epac2 reveal that in the absence of cAMP, the regulatory region covers the catalytic region and autoinhibits GEF activity by hindering the binding of Rap proteins to the

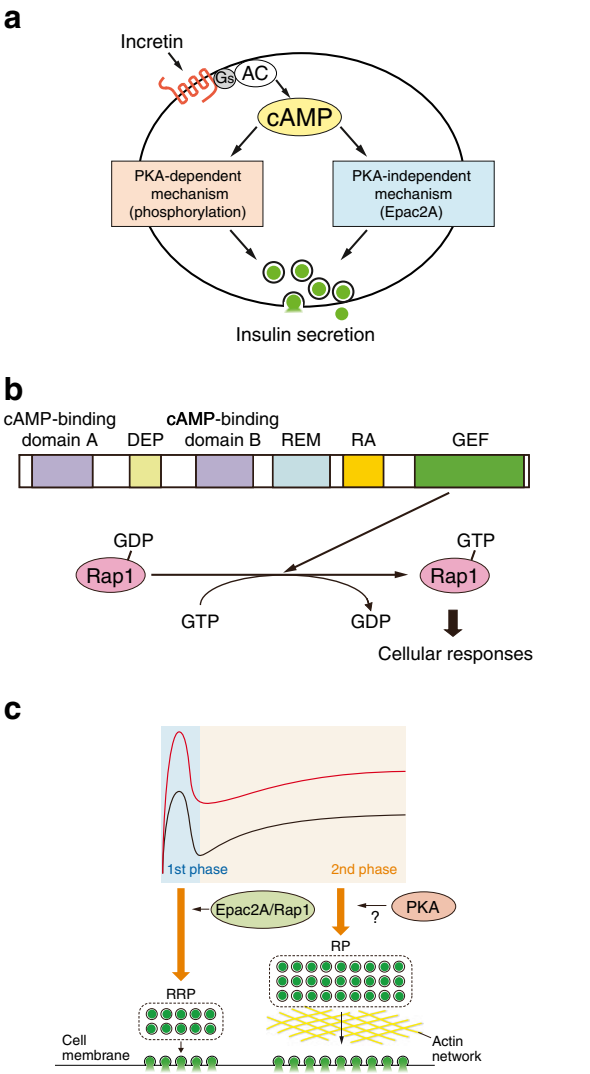
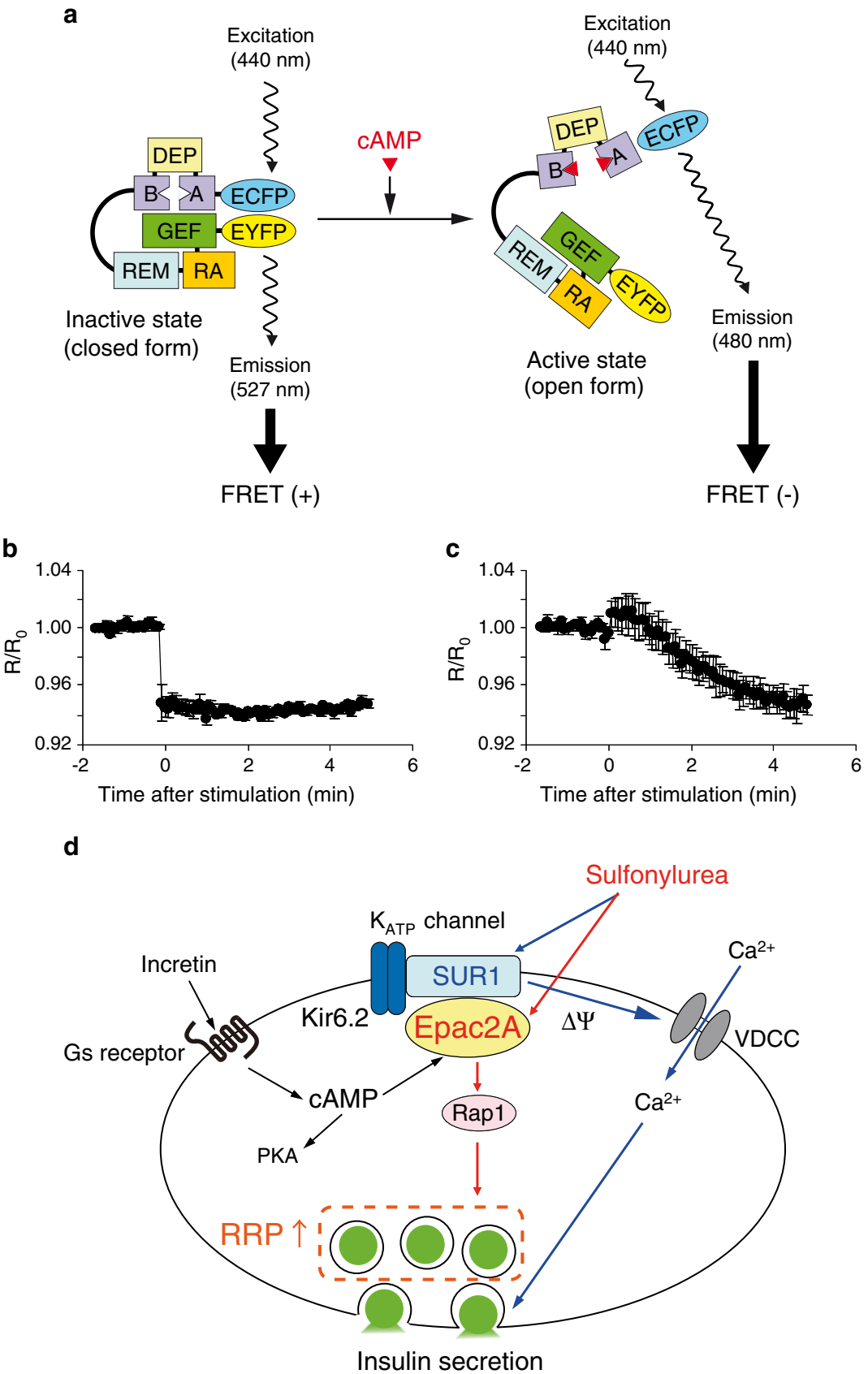


Fig. 4 **a** PKA-dependent and PKA-independent pathways of cAMP-potentiated insulin secretion. AC, adenylyl cyclase. **b** Functional domains of Epac2A (cAMP-GEFII). DEP, Dishevelled, Egl-10, and Pleckstrin; RA, Ras-association; REM, Ras exchange motif. **c** Proposed mechanism of potentiation of GIIS by cAMP signalling. Reproduced with permission of the American Society for Clinical Investigation, from [31]; permission conveyed through Copyright Clearance Center, Inc.

CDC25-homology domain [92]. Binding of cAMP to Epac causes a conformational change, thereby eliciting GEF activity toward Rap proteins [92]. Recently, a novel splicing variant was found in mouse adrenal glands [93]. This splicing variant, which lacks the amino-terminal cAMP-binding domain of Epac2, is designated Epac2B (adrenal type), while the original Epac2 is now called Epac2A (brain/beta cell type). A previously identified splicing variant, also lacking the amino-terminal cAMP-binding domain, which is specifically expressed in liver [94], is referred to as Epac2C (liver type). Conformational change of Epac1 induced by its binding to cAMP induces the translocation of Epac1 to the plasma membrane via the DEP domain [95]. Epac2A is localised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RRP ↑
Insulin secretion

Fig. 5 **a** Epac2A FRET sensor. A FRET sensor was constructed in which the full-length Epac2A was sandwiched between ECFP at the N-terminus and EYFP at the C-terminus. FRET occurs in the inactive state (closed form). Upon cAMP binding, Epac2A changes its conformation and becomes the open form. As a result, FRET does not occur. DEP, Dishevelled, Egl-10, and Pleckstrin; RA, Ras-association; REM, Ras exchange motif. **b**, **c** Activation of Epac2A by sulfonylureas. Tolbutamide (**b**) and glibenclamide (**c**) decrease Epac2A FRET, indicating that they activate Epac2A. The EYFP/ECFP ratio (R) was normalised to R_0 to describe FRET efficiency changes (FRET change=R/R_0), where R_0 is the EYFP/ECFP ratio at time 0. Reproduced from [103] with permission from AAAS. **d** Mechanisms of sulfonylurea action in insulin secretion. Closure of K_{ATP} channels is required for sulfonylureas to stimulate insulin secretion; activation of Epac2A/Rap1 signalling is required for sulfonylureas to exert their full effects on insulin secretion. $\Delta\Psi$, depolarising beta cell membrane, VDDC, voltage-dependent Ca^{2+} channel. Modified from [106] with permission from the Asian Association for the Study of Diabetes and Blackwell Publishing Asia Pty Ltd

to the plasma membrane through the interaction of the RA domain with activated Ras proteins [96, 97]. The amino-terminal cAMP-binding domain A of Epac2A also mediates its localisation to the plasma membrane [93]. Localisation of Epac2A to the plasma membrane is independent of its binding to cAMP.

Studies of Epac2A null (*Epac2a*$^{-/-}$) mice and Rap1 knockdown in clonal mouse beta cells indicate that Epac2A/Rap1 signalling is required for first phase potentiation of glucose-induced insulin granule exocytosis by cAMP [27]. It has been proposed that activation of Epac2A/Rap1 signalling increases the size of the RRP and/or recruitment of insulin granules from the RRP, while PKA signalling increases the size of the RP and/or recruitment of insulin granules from the RP (Fig. 4c) [31]. Rim2α has been found to be essential for Epac2A-mediated potentiation of GIIS by cAMP [43, 83]. Epac2A has also been shown to be involved in mobilisation of Ca^{2+} from intracellular Ca^{2+} stores in pancreatic beta cells [98]. The effect of Epac2A on Ca^{2+} mobilisation has been shown to be mediated by ryanodine receptors and IP_3 receptors by studying ryanodine receptor null mice and phospholipase Cε null mice, respectively [98].

cAMP signals are known to be compartmentalised in different regions of cardiac myocytes [99]. Such cAMP compartmentalisation is thought to underlie the distinct biological responses mediated by the different cAMP-increasing ligands. By analogy with the effects of cAMP in cardiac myocytes, cAMP compartmentalisation has also been proposed in pancreatic beta cells [84].

Epac2A as a target of sulfonylurea

Sulfonylureas stimulate insulin secretion by closing K_{ATP} channels through binding to SUR1, as mentioned above. Although sulfonylureas were also suggested to act intracellularly to stimulate insulin granule exocytosis [100–102], the direct target was not identified.

To screen for agents and ligands that activate Epac2A, Epac2A fluorescence resonance energy transfer (FRET) sensor, in which the full-length Epac2A was fused amino-terminally to enhanced cyan fluorescent protein (ECFP) and carboxyl-terminally to enhanced yellow fluorescent protein (EYFP; termed C-Epac2A-Y) was established (Fig. 5a) [103]. FRET is the radiationless transfer of energy from an initially excited donor to an acceptor [104]. It is dependent on the proper spectral overlap of the donor and acceptor, their distance from each other and the relative orientation of the chromophore's transition dipoles. In the case of green fluorescent proteins (GFPs), including ECFP and EYFP, the distance between a donor and an acceptor must be within 5 nm for FRET to be detectable [105]. Epac2A is a closed form in the inactive state, so that ECFP and EYFP are located very close to each other, which causes FRET. Upon binding of cAMP,

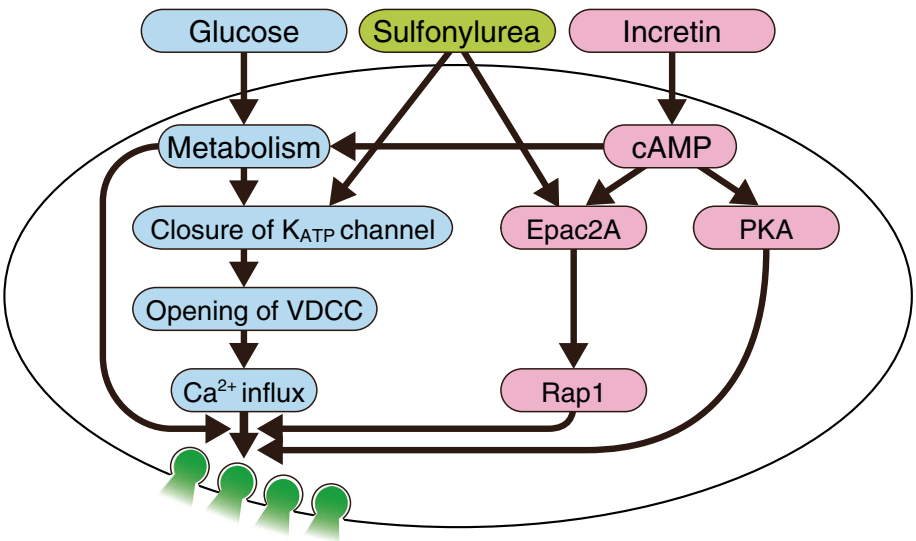

Fig. 6 Model of interaction of glucose, incretin and sulfonylurea. VDCC, voltage-dependent Ca^{2+} channel

Epac2A changes its conformation, so that ECFP and EYFP separate away. As a result, FRET does not occur (active state). Utilising the Epac2A FRET sensor, the activation status of Epac2A can be monitored. In the course of screening for agents that activate Epac2A, we found that tolbutamide and glibenclamide, both of which are glucose-lowering sulfonylurea drugs, significantly decreased the FRET response in C-Epac2A-Y transfected cells [103] (Fig. 5b, c). The effect on FRET was confirmed using other sulfonylureas, including chlorpropamide, acetohexamide, and glipizide, all of which significantly decreased the FRET response to different degrees and with varying kinetics. However, gliclazide, another sulfonylurea, did not change FRET. Glinide derivatives, which stimulate insulin secretion by closure of K_{ATP} channels by acting directly on SUR1, had no effect on the FRET response. Direct binding of sulfonylurea to Epac2A was confirmed by specific binding of radiolabelled glibenclamide to Epac2A expressed in COS-1 cells. In addition, the sulfonylureas that decreased FRET all activated Rap1 in MIN6 cells, whereas gliclazide did not. These results indicate that Epac2A is a target of sulfonylurea. Moreover, tolbutamide-induced insulin secretion and glibenclamide-induced insulin secretion from isolated pancreatic islets were significantly reduced in Epac2A null mice compared with wild-type mice. However, there was no significant difference in insulin secretion in response to gliclazide. Furthermore, the insulin response to oral administration of tolbutamide alone or to concomitant administration of glucose and tolbutamide in Epac2A null mice was significantly reduced compared with that in wild-type mice, and the glucose-lowering effect of tolbutamide in Epac2A null mice was significantly less than that in wild-type mice. Although closure of the K_{ATP} channels is essential for sulfonylureas to stimulate insulin secretion, activation of Epac2A/Rap1 signalling is required for sulfonylureas to exert their full effects with regard to insulin secretion (except for gliclazide). Considering the role of Epac2A/Rap1 signalling in insulin granule exocytosis [27], sulfonylureas may increase the size of the RRP of insulin granules near the plasma membrane (Fig. 5d) [106]. Since Epac2A is also the target of incretin/cAMP signalling, Epac2A is critical for the actions of both sulfonylureas and incretin-related glucose-lowering drugs such as DPP-4 inhibitors and GLP-1 receptor agonists. It is possible that sulfonylurea and cAMP signalling interact with each other through Epac2A, in which case the effects of sulfonylurea on Epac2A might be influenced by cellular cAMP concentrations and vice versa.

Conclusions

GIIS is the principal mechanism of insulin secretion. Potentiation of GIIS by cAMP-increasing ligands such as incretin is also critical for normal regulation of insulin secretion. Sulfonylurea, a glucose-lowering drug widely used for treatment of diabetes, stimulates insulin secretion. Utilising molecular biology and gene technology, it has been shown that the K_{ATP} channel is the target of both ATP, an essential signal for GIIS, and sulfonylurea, and that Epac2A is the target of both cAMP, a critical signal for potentiation of GIIS, and sulfonylurea. It is now known that ATP, cAMP, and sulfonylurea interact with one another to facilitate stimulus–secretion coupling in insulin release (Fig. 6). In 1970, Prof. Albert Renold published the article ‘Insulin biosynthesis and secretion – a still unsettled topic’ in *The New England Journal of Medicine* [107]. Although our understanding of insulin secretion has increased remarkably since that time, the title of the paper remains true.

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