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Epac2 signaling at the β-cell plasma membrane

IDA ALENKVIST

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Abstract

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Secretion of appropriate amounts of insulin from pancreatic β-cells is crucial for glucose homeostasis. The β-cells release insulin in response to glucose and other nutrients, hormones and neurotransmitters, which trigger intracellular signaling cascades, that result in exocytotic fusion of insulin-containing vesicles with the plasma membrane. Increases of the intracellular concentration of calcium ions ($[Ca^{2+}]_i$) trigger exocytosis, whereas the messenger cyclic adenosine monophosphate (cAMP) amplifies various steps of the secretion process. The protein Epac2 mediates some effects of cAMP, but little is known about its regulation in β-cells. In this study, the spatio-temporal dynamics of Epac2 was investigated in insulin-secreting MIN6-cells and primary β-cells using various cell signaling biosensors and live-cell fluorescence microscopy approaches. Increases in the cAMP concentration triggered translocation of Epac2 from the cytoplasm to the plasma membrane. Oscillations of cAMP induced by glucose and the insulin-releasing hormone GLP-1 were associated with cyclic translocation of Epac2. Analyses of Epac2 mutants showed that the high-affinity cyclic nucleotide-binding domain and Ras-association domains were crucial for the translocation, whereas neither the DEP domain, nor the low-affinity cAMP-binding domain were required for membrane binding. However, the latter domain targeted Epac2 to insulin granules at the plasma membrane, which promoted their priming for exocytosis. Depolarization-induced elevations of $[Ca^{2+}]_i$ also stimulated Epac2 translocation, but the effects were complex and in the presence of high cAMP concentrations, $[Ca^{2+}]_i$ increases often reduced membrane binding. The stimulatory effect of Ca^{2+} was mediated by increased Ras activity, while the inhibitory effect reflected reduced concentrations of the membrane phospholipid PtdIns(4,5)P_2. Anti-diabetic drugs of the sulfonylurea class, suggested to directly activate Epac2, induced translocation indirectly by depolarizing β-cells to increase $[Ca^{2+}]_i$. Epac2 is an activator of Rap GTPases, and its translocation increased Rap activity at the plasma membrane. It is concluded that the subcellular localization of Epac2 is controlled by a complex interplay between cAMP, Ca^{2+} and PtdIns(4,5)P_2 and that the protein controls insulin release by binding to the exocytosis machinery. These results provide new insights into the regulation of β-cell function and may facilitate the development of new anti-diabetic drugs that amplify insulin secretion.

Keywords: β-cell, insulin secretion, cAMP, Ca2+, Epac, Rap, exocytosis, sulfonylurea

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Idevall-Hagren, O., **Jakobsson, I***., Xu, Y., and Tengholm, A. (2013) Spatial control of Epac2 activity by cAMP and Ca^{2+}-mediated activation of Ras in pancreatic β cells. *Science Signaling* 6, ra29.1-11, S1-6. doi 10.1126/scisignal.2003932
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II **Alenkvist, I**., Gandasi, N., Barg, S., and Tengholm, A. (2016) Two-step membrane recruitment of Epac2 primes secretory granules for exocytosis. *Manuscript*

III **Alenkvist, I**., Idevall-Hagren, O., and Tengholm, A. (2016) Localization of Epac2 to the plasma membrane is controlled by an interplay between cAMP, Ca^{2+} and PtdIns(4,5)P_2. *Manuscript*

IV **Alenkvist, I**., Xu, Y., and Tengholm, A. (2016) Sulfonylureas promote Ca^{2+}-dependent Epac2 plasma membrane binding and Rap activation. *Manuscript*

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Abbreviations

AC	Adenylyl cyclase
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine 3´,5´-monophosphate
CFP	Cyan fluorescent protein
CNB1	The low-affinity cAMP-binding domain of Epac2
CNB2	The high-affinity cAMP-binding domain of Epac2
DEP	Disheveled, Egl-10, and pleckstrin
Epac	Exchange protein directly activated by cAMP
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GLP-1	Glucagon-like peptide-1
IBMX	3-isobutyl-1-methylxantine
IP_3	inositol-1,4,5-trisphosphate
K_{ATP} channel	ATP-sensitive K^+ channel
NPY	Neuropeptide Y
PDE	Phosphodiesterase
PH	Pleckstrin homology
PIP	Phosphatidylinositol 4-phosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
RA	Ras association
REM	Ras-exchange motif
SUR1	Sulfonylurea receptor 1 subunit on the K_{ATP} channel
TIRF	Total internal reflection fluorescence
VDCC	Voltage-dependent Ca^{2+} channel
WT	Wildtype
YFP	Yellow fluorescent protein

Introduction

The blood glucose concentration is maintained in a rather narrow range despite large variations in energy intake and consumption that occurs as a consequence of e.g. eating, exercise and overnight fasting. Because glucose is an indispensable nutrient for the brain, very low blood glucose levels are acutely life threatening. High blood glucose concentrations, on the other hand, are no acute danger, but have long-term adverse effects on many tissues and organs. Blood glucose control is therefore very important and this function is exerted by interplay between several tissues and organs, including the pancreas, liver, brain, muscle and fat [1,2].

The pancreas is a prime regulator of glucose homeostasis by producing and releasing blood-glucose-regulating hormones. The majority of the gland (99%) is exocrine and produces digestive juices and only a very small fraction (1%) is endocrine and releases hormones into the circulation. The endocrine part consists of groups of up to a few thousand cells called islets of Langerhans [3]. The islets contain five different endocrine cell types: α-, β-, δ-, ε- and PP-cells. About 60-80% of the endocrine islet cells in the mouse are β-cells, which produce and release insulin in response to increasing blood glucose levels, other nutrients, hormones and neural factors. Insulin lowers the blood glucose by promoting glycogen formation and suppressing glycogenolysis and gluconeogenesis in the liver as well as by promoting glucose uptake and storage in muscle and adipose tissue [1]. The β-cell is the major culprit in most forms of diabetes, which is characterized by deficient insulin secretion with resulting hyperglycemia. The second most abundant cell type in the islets is the α-cell (20-30% in the mouse), which secretes glucagon under hypoglycemic conditions [4]. Glucagon restores normoglycemia mainly by stimulating glucose release from the liver. δ-cells release somatostatin, which is believed to regulate the release of the other hormones by local action in the islet [5]. A very small fraction of cells are pancreatic polypeptide-secreting PP-cells [6] and ghrelin-releasing ε-cells [7]. Numerous effects have been ascribed to these hormones but they seem to lack direct influence on blood glucose regulation.

The present study focuses on the β-cell and the mechanisms that underlie the regulation of insulin secretion. The β-cells responding factors typically trigger intracellular signaling cascades that involve different second messengers and signaling proteins. This thesis deals with Epac2, a signaling protein that is activated by the second messenger cAMP, which is involved in the regulation of insulin secretion by e. g. glucose and the gut hormone glucagon-like peptide-1 (GLP-1).

Background

Insulin secretion kinetics

Glucose is a major stimulus of insulin secretion. When β-cells are stimulated with glucose, there is a lag of up to a few minutes before secretion is initiated. This delay reflects the time required for metabolism to generate sufficient ATP to depolarize the plasma membrane to trigger voltage-dependent Ca^{2+} influx [8]. The initial secretory response measured from groups of islets, the whole pancreas or *in vivo*, is named first phase insulin secretion. It is pronounced and lasts for a few minutes. The subsequent second phase secretion is less prominent but maintained [9,10].

The biphasic nature of insulin secretion has been explained by the involvement of different pools of granules [9,11,10]. A small fraction (~1%) of the approximately 10.000 granules in a β-cell belongs to a readily releasable pool docked to the plasma membrane, which can undergo exocytosis very quickly in response to Ca^{2+} elevation [12]. The remaining granules belong to a reserve pool that need to undergo docking and a series of maturation steps, named priming, before they can be released. First phase insulin secretion has been suggested to reflect release of the readily-releasable granule pool, and the second phase release of granules recruited from the reserve pool [11,13]. Detailed measurements of insulin release kinetics in islets or single cells, show that the second phase of insulin secretion consists of distinct pulses, that are similar to the first phase pulse but less pronounced [14-19]. There are also oscillations of insulin in the portal vein and peripheral circulation *in vivo* [20-22] indicating that pulsatile secretion must be coordinated among β-cells within and between the islets in the pancreas. Insulin oscillations increase the action of the hormone on the liver [23]. Interestingly, disturbed insulin pulsatility is a hallmark of early stages of diabetes [22].

Beside glucose and its direct effects of on the β-cells, food intake stimulates insulin secretion indirectly by triggering hormone release from endocrine cells in the gut. GLP-1 is one such hormone that is released from the neuroendocrine L-cells in the intestinal mucosa in response to dietary nutrients, especially carbohydrates and fat [24]. GLP-1 acts on β-cells via G-protein-coupled receptors to elevate cAMP, which increases electrical activity and Ca^{2+} signaling and amplifies the exocytotic release of the insulin secretory vesicles [25,26]. Glucose-dependent insulinotropic polypeptide (GIP) is another incretin hormone released from the intestinal K-cells in response to dietary nutrients, especially carbohydrates and fat. GIP has similar effect as GLP-1 on the β-cell [26] and the combined stimulatory incretin effect on insulin secretion is about 50-70%.

Stimulus-secretion coupling in the β-cell

The stimulus-secretion-coupling process (Fig. 1) involves glucose entry into the β-cell through GLUT transporters [27] followed by metabolism in glycolysis, the mitochondrial tricarboxylic acid cycle and the electron transport chain, which results in ATP generation [28,29]. The increase of the ATP/ADP ratio leads to closure of ATP-sensitive K^+ (K_{ATP}) channels in the plasma membrane and membrane depolarization. When depolarization reaches the activation threshold for voltage-dependent Ca^{2+} channels (VDCCs), the ion rapidly enters the β-cell [30]. The increase of the cytoplasmic Ca^{2+} concentration triggers release of the insulin containing secretory granules [30]. The L-type VDCCs Ca^{2+} channels are most important for insulin secretion [31]. These channels bind to the secretory granules by SNARE proteins to enable the local Ca^{2+} concentration to reach high levels [13]. The glucose-induced Ca^{2+} response is often periodic, and several types of Ca^{2+} oscillations have been described in individual β-cells and intact pancreatic islets [32-35,5]. The dominating type has a frequency of 0.3-0.9/min. β-cells in intact islets often show more rapid oscillations (2-7/min) that in addition to voltage-dependent Ca^{2+} entry involves release of Ca^{2+} from the intracellular stores [36]. The K_{ATP} channel is the target for the sulfonylurea class of drugs, which is widely used for treatment of type 2 diabetes. Sulfonylureas depolarize β-cells and induce Ca^{2+} influx and insulin secretion by inhibiting the K^+ channel by binding to its accessory subunit SUR1 (sulfonylurea receptor 1) [37-39].

By energizing the sarco/endoplasmic reticulum Ca^{2+}-ATPase, glucose-generated ATP also promotes uptake of Ca^{2+} into the endoplasmic reticulum (ER) [40]. This Ca^{2+} store can be mobilized by various stimuli that activate phospholipase C (PLC), an enzyme that catalyzes formation of the Ca^{2+}-mobilizing messenger inositol-1,4,5-trisphosphate (IP_3) [35] by hydrolysis of the plasma membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2 or PIP_2) [41]. IP_3 activates receptors in the ER, which triggers release of Ca^{2+} into the cytoplasm. PIP_2 hydrolysis also results in formation of the signaling lipid diacylglycerol, which among other functions activates protein kinase C [42]. In addition to being a precursor for second messengers, PIP_2 has functions on its own as a regulator of ion channels and transporter proteins and as a membrane-localized binding site that can recruit and activate signaling proteins [43].

Glucose stimulates insulin secretion also by generating metabolic factors, that amplify exocytosis at steps distal to Ca^{2+} [44]. The nature of this amplifying pathway is not entirely clear and several factors have been discussed in this context, including adenine and guanine nucleotides, protein kinases, long chain acyl-CoA, glutamate and cAMP [30]. Glucose stimulates the formation of cAMP in β-cells [45,16,46,47]. Although cAMP is not the only metabolic amplifying signal [48], the glucose-induced cAMP formation is crucial for an optimal insulin secretory response. The intracellular cAMP concentration oscillates in glucose-stimulated β-cells and the oscillations are largely in phase with those of Ca^{2+} [16,47]. The coordinated oscillations of Ca^{2+} and cAMP generate pulsatile insulin release [16,18].

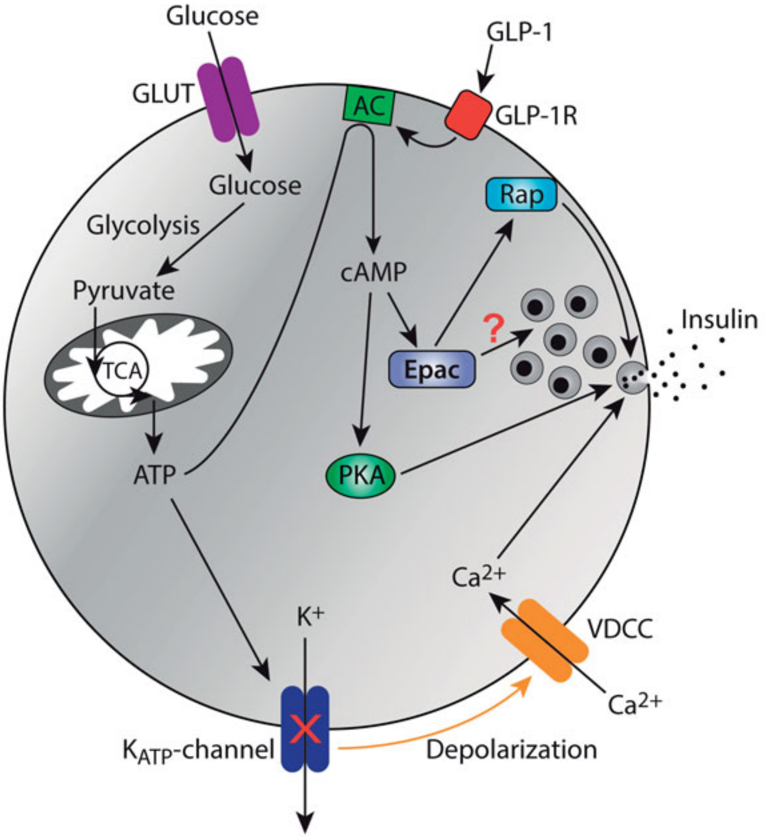

Figure 1: *Glucose- and GLP-1-stimulated insulin secretion in β-cells.* Glucose enters the β-cell via GLUT transporters and subsequent glycolysis and oxidative mitochondrial metabolism result in increased ATP levels. ATP closes the ATP sensitive K^+ (K_{ATP}) channels, which lead to membrane depolarization Ca^{2+} influx through VDCCs and exocytosis of insulin secretory granules. Glucose metabolism also promotes cAMP generation by stimulating adenylyl cyclases (AC). GLP-1 amplifies glucose-induced insulin secretion by binding to its receptor, which leads to activation of AC and cAMP formation. cAMP amplifies insulin secretion via PKA and Epac. Both effectors act at on the exocytosis machinery but the detailed mechanisms are incompletely understood. At least some effects of Epac involve activation of the small GTPase Rap.

cAMP signaling

Adenylyl cyclases

cAMP is a ubiquitous second messenger involved in many different cellular signaling processes. cAMP is formed from ATP by the adenylyl cyclase (AC) group of enzymes. There are ten different isoforms of ACs in mammalian cells, which differ somewhat in structure and regulatory properties [49]. Nine isoforms are transmembrane proteins [49] and they are typically activated by G-protein-coupled receptors, such as the GLP-1 receptor, by interaction with the Gαs subunit of the receptor-associated heterotrimeric G protein. The activity of some ACs is stimulated by Ca^{2+}, which allows crosstalk between the Ca^{2+} and cAMP signaling pathways [50]. AC8 is an example of a Ca^{2+}-activated AC, which has been proposed to play an important role in integrating glucose and hormone receptor stimuli in β-cells [51]. There are also Ca^{2+}-inhibitable ACs, including AC5, which was recently found to be important for glucose-induced insulin secretion in human β-cells [52]. Most of the transmembrane ACs can be activated by the diterpene forskolin [49], which is a common experimental tool for elevating intracellular cAMP. One AC isoform, AC10 or soluble AC, does not have the transmembrane structure. It is not activated by Gαs and therefore does not mediate receptor-induced cAMP elevations [53]. Due to its sensitivity to bicarbonate, ATP and Ca^{2+}, AC10 has been implicated as a metabolic sensor [54,55], but the importance of AC10 in β-cells remains uncertain [47].

Phosphodiesterases

cAMP is degraded by cyclic nucleotide phosphodiesterases (PDEs). This is a large group of enzymes divided into eleven different families based on structural and regulatory features and substrate specificity [56]. Only three of the PDE families specifically hydrolyze cAMP, whereas others also degrade cyclic guanosine monophosphate (cGMP) or are entirely cGMP-specific [56]. β-cells express several PDE family members, where PDE1, PDE3, PDE4 and PDE8 seems to be most important for shaping the cAMP signals [57-59]. PDE1 can also become activated by increasing levels of Ca^{2+} [57]. Most PDEs can be inhibited by the 3-isobutyl-1-methylxantine (IBMX), which is often used experimentally for elevating intracellular cAMP levels.

cAMP effectors

Increasing cAMP concentrations activate protein kinase A (PKA) and the guanine nucleotide exchange factor (GEF) Epac, also known as cAMP-GEF [60-62]. The PKA holoenzyme consists of two regulatory subunits, each associated with a catalytic subunit. There are four isoforms of the regulatory subunit (RIα, RIβ, RIIα and RIIβ) as well as of the catalytic subunit (Cα, Cβ, Cδ and PrKX) [63,60]. PKA holoenzymes based on RI regulatory subunits, referred to as type I, are mainly cytosolic, whereas RII-based PKA (type II) often interacts with specific binding proteins, A-kinase anchoring proteins (AKAPs) [60], located at various intracellular membranes, such as the plasma, nuclear and secretory granule membranes [64]. Two cAMP binding sites are present on each regulatory subunit with PKA type I showing somewhat higher affinity (with activation at 50-100 nM) than PKA type II (with activation at 200-400 nM) [60,65]. The binding of cAMP induces a conformational change that results in dissociation of the holoenzyme [60]. The free monomeric catalytic subunits can then regulate different cellular processes by phosphorylating various protein substrates [64]. PKA is involved in phosphorylate several proteins in the insulin secretion pathway. Both the SUR1 and Kir6.2 subunits of the K_{ATP} channel are phosphorylated by PKA. Phosphorylation of SUR1 makes the channel more prone to depolarize, whereas Kir6.2 phosphorylation increasing the channel activity [66,67]. PKA phosphorylates the L-type VDCC and enhances Ca^{2+} influx [68,67]. The kinase also sensitizes the IP_3 receptor to release Ca^{2+} into the cytoplasm [69,67]. In addition, several exocytosis proteins like SNAP-25, RIM1α, synapsin and snapin are phosphorylated [67].

Epac

PKA was for a long time thought to be the only cAMP effector in most cells. Evidence for the presence of PKA-independent effects of cAMP in β-cells was obtained in 1997 from studies of exocytosis, in which a significant stimulatory effect of cAMP remained in the presence of PKA inhibitors [70]. In 1998, two research groups independently discovered a cAMP-regulated GEF, which was named cAMP-GEF or Epac (Exchange protein directly activated by cAMP) [71,72].

Epac is involved in many cAMP regulated processes, like cell adhesion, cell-cell junction formation, exocytosis, phagocytosis, differentiation, proliferation, gene expression and apoptosis [73-77]. There are two isoforms of the protein, Epac1 and Epac2, that are encoded by separate genes [72]. Both isoforms are specific guanine nucleotide exchange factors (GEFs) of the small GTPases Rap1 and Rap2, where they stimulate GTP/GDP exchange and thereby activate them [78]. Epac1 is ubiquitously expressed, while Epac2 has a more restricted tissue distribution with expression in the brain, pituitary, pancreatic islets, adrenals, and the liver [71,72,79,80]. Of three different Epac2 splice versions, the full-length Epac2A is the most prominent variant in islets [80].

Soon after the discovery of Epac, it was demonstrated that the protein mediates the PKA-independent effects of cAMP on exocytosis [73]. Various observations have subsequently confirmed the involvement of Epac in insulin secretion. For example, pharmacological activation of Epac with cyclic nucleotide analogues amplifies insulin secretion from clonal β-cells and islets [81-84]. Mice deficient in Epac2 show normal glucose homeostasis but reduced insulin secretion in response to cAMP-elevating agents [85] and after metabolic challenge of the β-cells by high fat feeding [86]. Also a homozygous Epac1 knockout mouse develops an impaired glucose tolerance [87].

The Epac proteins are multi-domain proteins, which consist of an N-terminal regulatory region and a C-terminal catalytic region (Fig. 2). The regulatory region contains one cyclic-nucleotide-binding domain in Epac1 and two such domains in Epac2 (CNB1 and CNB2). There is also a Dishevelled/Egl-10/pleckstrin (DEP) domain, which is located N-terminal of the cyclic-nucleotide-binding domain in Epac1 and between CNB1 and CNB2 in Epac2. The DEP domain is involved in plasma membrane targeting of Epac1 [88] but its role in Epac2 is uncertain [89]. In the catalytic region, both isoforms contain a Ras-exchange motif (REM), which is supposed to stabilize the catalytic domain, and a Ras association (RA) domain, which in Epac2 has been found to interact with the Ras GTPase [89]. There is also the catalytic CDC25-homology domain (CDC25HD), which is the part of the protein that houses the GEF activity (Fig. 2) [78,90,91]. The single cyclic-nucleotide-binding domain in Epac1 and CNB2 in Epac2, bind cAMP with an order of magnitude lower affinity than the isolated cAMP-binding domains in PKA, but in the same range as the PKA holoenzyme [92,65]. Moreover, CNB1 has approximately 70-fold lower affinity for cAMP than CNB2 in Epac2 [92] but the functional importance of cAMP binding to CNB1 is unclear. Crystal studies of Epac2 have demonstrated that in the inactive form, the regulatory region is folded over the catalytic region to prevent access of the active site to its substrate [90]. When cAMP binds to the CNB2 domain there is a large conformational change that relieves the auto-inhibition and allows access of the catalytic region to the substrate [93].

It was recently proposed that sulfonylureas, in addition to their well-known effect on the K_{ATP} channel, acts by direct binding to and activation of Epac2 [94,95]. This idea was based on experiments with a fluorescent Epac2 activity reporter, in binding studies with radioactively labeled sulfonylureas, as well as on the observation that sulfonylureas activate Rap activity. The drug supposedly binds cooperatively with cAMP to the CNB1 domain of Epac2 [96]. However, several other groups have failed to find support for these conclusions [97,98] and the role of Epac2 as a sulfonylurea receptor remains an open question.

The detailed mechanisms of action of Epac in insulin secretion have not yet been clarified. Epac2 has been suggested to promote insulin secretion by stimulating secretory granule recruitment to the plasma membrane, an effect that might be mediated by Rap1 [85]. Epac2 has also been suggested to activate Ca^{2+} signaling via phospholipase C-ε [99,100]. There is evidence for a direct interaction between Epac2 and the SUR1 subunit of the K_{ATP} channel [73]. This interaction may be important for the effect of Epac2 on secretion, because PKA-independent stimulation of exocytosis by cAMP is absent in SUR1-deficient mice [101]. In addition Epac2 has been found to interact with several other proteins involved in the cellular exocytosis machinery [102-104].

The Epac downstream target proteins Rap1 and Rap2 have been found to localize to various membrane compartments, including the Golgi network, the nuclear membrane, vesicular membranes and the plasma membrane [105-107]. Activation of proteins at specific sites might be achieved by subcellular targeting of their upstream GEFs. The Epac proteins have indeed been found both at the plasma membrane and at various organelle membranes but the mechanisms underlying the subcellular distribution of Epac proteins are not well understood. Increases in the cAMP concentration has been found to trigger Epac1 translocation to the plasma membrane [108]. The membrane binding involves the DEP domain, which interacts with phosphatidic acid [88], as well as the N-terminal part of the protein, which interacts with activated ezrin-radixin-moesin (ERM) proteins [109]. The present thesis focuses on the spatiotemporal regulation of Epac2 and the functional role of the protein in insulin-secreting β-cells.

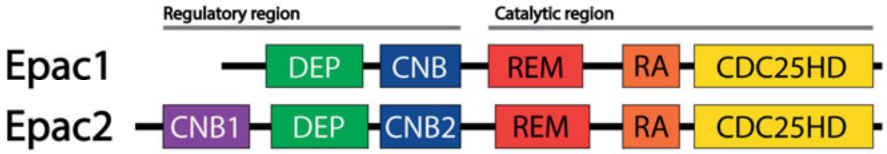

Figure 2: *Structure of the Epac proteins.* The two Epac isoforms have a similar structure with a regulatory and a catalytic region, each containing multiple domains. CNB, cyclic nucleotide binding domain. Epac1 has one, whereas the longest splice version of Epac2 (Epac2A) has two CNB domains. DEP, Dishevelled/Egl-10/pleckstrin domain; REM, Ras-exchange motif; RA, Ras association domain; CDC25HD, CDC25-homology domain.

Aims

The aims for this study were to:

1. Elucidate how the subcellular distribution of Epac2 in β-cells is influenced by cAMP and various insulin secretagogues. (paper I, III)

2. Investigate how the different domains in the Epac2 protein are involved in the targeting to the plasma membrane. (paper I, II and III)

3. Clarify how the localization of Epac2 is related to sites for insulin secretory granule docking and exocytosis. (paper II)

4. Investigate how spatial redistribution of Epac2 influences Rap activity at the plasma membrane. (paper I)

5. Clarify the potential link between sulfonylureas and Epac2 activity. (paper IV)

Experimental procedures

MIN6 β-cells and pancreatic islets

The murine insulin-secreting β-cell line MIN6 [110] was used in most experiments in this thesis. This cell line responds to glucose with several-fold stimulation of insulin secretion. To avoid potential phenotypic changes with increasing passage number all experiments were performed with cells within a narrow range of low passages (17-30). For live-cell imaging experiments, the cells were transfected with plasmids for various fluorescent reporter constructs and seeded onto coverslips.

The major findings were reproduced in primary β-cells. Pancreatic islets were isolated from 5-7 months old C57Bl/6J mice and cultured in RPMI 1640 medium containing 5.5 mM glucose. For some experiments human islets from cadaveric organ donors were obtained via the Nordic Network for Clinical Islet Transplantation. These islets were cultured in CMRL 1066 medium with 5.5 mM glucose. Islets from both species were transduced with adenoviral vectors expressing the fluorescent reporter constructs. For some experiments, the islets were dispersed into single cells and small clusters before seeded onto coverslips.

Imaging Epac2 and active Rap and Ras location

To visualize the subcellular location of Epac2, the cells or islets were transfected or transduced with vectors encoding Epac2A with an N-terminal GFP or mCherry tag. Epac2 mutants lacking specific domains or carrying critical amino acid substitutions were employed to elucidate how the subcellular localization is determined (Fig. 3). Experiments were also employed with an isolated CNB1 domain from Epac2 and with full-length Epac1 tagged with fluorescent protein. The various constructs were either obtained from other investigators [89] or generated by PCR and site-directed mutagenesis. Fluorescence was recorded with confocal or total internal reflection fluorescence (TIRF) microscopy (see below).

A GFP-labeled Rap-binding domain from the protein RalGDS, which selectively binds to the GTP-bound form of Rap [111] was used for detection of active Rap. When monitoring plasma membrane fluorescence with TIRF microscopy, intensity changes represent alterations in the protein binding to the plasma membrane, which directly reflects the activity of Rap at the membrane. Ras activity was monitored by a similar approach using the Ras-binding domain from the protein Raf1$_{RBD}$ tagged with CFP [112].

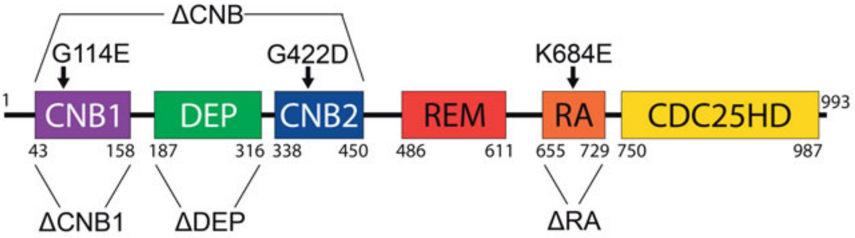

Figure 3: *Epac2 constructs used in the present study.* Schematic illustration of the Epac2 domain structure, highlighting domains that have been deleted and amino acids that have been substituted in different constructs. The numbers refer to the position of amino acid residues. ΔCNB indicates deletion of both cyclic nucleotide binding (CNB) domains and the intervening DEP (Disheveled, Egl-10, Pleckstrin) domain, ΔRA indicates deletion of the Ras association (RA) domain, ΔCNB1 indicates the deletion of the CNB1 domain, ΔDEP indicates the deletion of the DEP domain, G114E indicates a glycine to glutamic acid substitution in the low-affinity cAMP-binding site at amino acid position 114 of the full length protein, G422D indicates a glycine to aspartic acid substitution in the high-affinity cAMP-binding site at amino acid position 422, and K684E indicates a lysine to glutamic acid substitution in the Ras-binding site at amino acid position 684.

Measurements of the Ca^{2+} and cAMP concentrations beneath the plasma membrane

The cytoplasmic Ca^{2+} concentration was measured in cells loaded with the fluorescent Ca^{2+} indicators Fluo-4 or Fura Red, or transfected with the genetically encoded fluorescent protein based sensors GCaMP5G [113] or R-GECO [114]. Binding of Ca^{2+} induces changes in fluorescence intensity of these indicators and sensors. Fluorescence detection was restricted to the sub-plasma membrane space with TIRF microscopy.

The sub-plasma membrane concentration of cAMP was recorded with a translocation biosensor consisting of a plasma membrane-anchored and truncated form of the PKA RIIβ regulatory subunit and a YFP or mCherry-labeled PKA Cα catalytic subunit [115]. When the cAMP concentration beneath the plasma membrane increases, the catalytic subunit dissociates from the regulatory subunit at the membrane and diffuses into the cytoplasm. This translocation is detected with TIRF microscopy as loss of fluorescence from the plasma membrane. In some experiments, a CFP-tagged regulatory subunit was employed, and since it is permanently anchored at the plasma membrane, the CFP signal was used as reference in ratiometric recordings of cAMP.

Single-cell recordings of insulin secretion

Insulin secretion dynamics was monitored in individual MIN6 β-cells by recording the phosphoinositide 3-kinase-mediated formation of the phospholipid PIP_3 in the plasma membrane, which closely reflects the autocrine activation of insulin receptors [116]. PIP_3 was detected with the pleckstrin homology (PH) domain from Akt fused to YFP and fluorescence recorded with TIRF microscopy.

Measurements and manipulation of PIP_2

To measure PIP_2 at the plasma membrane the cells were transfected with the PH domain of phospholipase C δ1 (PLCδ1) tagged with fluorescent protein. This sensor binds PIP_2 with high selectively over other phosphoinositide lipids [117] and its binding to the plasma membrane was monitored with TIRF microscopy.

The PIP_2 concentration at the plasma membrane was rapidly lowered on demand by light-controlled membrane recruitment of a lipid phosphatase. Advantage was taken of the blue light-induced dimerization between the plant proteins cryptochrome 2 (CRY2) and the N-terminal part of the transcription factor CIB1 (CIBN). CIBN is anchored to the plasma membrane by a transmembrane domain and CRY2 is located in the cytoplasm in the dark state. CRY2 was fused to the inositol 5-phosphatase domain of OCRL (Lowe oculocerebrorenal syndrome protein), which hydrolyzes PIP_2 to phosphatidylinositol 4-phosphate. When exposed to blue light (from a 491 nm imaging laser) a FAD molecule bound to the photolyase homology region of CRY2 absorbs a photon. This triggers a conformational change in the domain that promotes binding to CIBN. The CRY2-5-phosphatase thereby translocates to the plasma membrane and hydrolysis PIP_2 [118,119]. The translocation is rapidly reversible when the light is turned off.

Imaging secretory granule docking and exocytosis and analysis of protein localization at the granule sites

Insulin granules were visualized in cells transfected with fluorescent-protein-tagged neuropeptide Y (NPY), which sorts into insulin secretory vesicles [120]. TIRF microscopy was used to image granules in the immediate vicinity of the plasma membrane. Exocytosis was observed as rapid disappearance of the granule fluorescence.

The localization of fluorescence-labeled Epac2 at secretory granules was quantified. Granules that were well separated from other granules or the edge of the cell were identified, and their locations transferred to the Epac2 spectral channel. An algorithm implemented in the MetaMorph software read the average pixel fluorescence in a central circle, a surrounding annulus and a background area not containing any cells. Since the granule site is smaller than the resolution of the microscope, the circle will contain all of the fluorescence originating from the granule. It also contains fluorescence from molecules not bound to the granule site. To obtain the specific on-granule fluorescence (ΔF), the annulus value was subtracted from that of the circle. To obtain off-granule fluorescence (S), the annulus value was background-corrected. The ratio between the on- and off-granule intensities (ΔF/S) is a convenient measure of binding to the granule site, independent of the expression level. Positive ratios indicate enrichment at the granule site and negative values indicate exclusion.

TIRF microscopy

Fluorescence within less than 100 nm from the plasma membrane adhering to the coverslip was recorded with TIRF microscopy. One setup was custom-built and based on an upright microscope contained in an acrylic glass box thermostated at 37 °C. Lasers provided 442 nm light for excitation of CFP, 488 and 514 nm for GFP and YFP, respectively, and 561 nm for the red fluorescent proteins td2 and mCherry. The laser beams were merged with dichroic mirrors, homogenized and expanded before being focused through a dove prism to achieve a 70° angle of the excitation light. In dual channel recordings the laser lines were selected with interference filters in a filter wheel. Fluorescence from the cells was collected through a 40x 0.8 numerical aperture (NA) water immersion objective and detected at 530/50 nm for GFP, 485/25 nm for CFP, and 542/27 nm for YFP or 625LP for td2/mCherry/Fura Red with a CCD camera.

An alternative TIRF imaging setup based on an inverted microscope was used for experiments involving intact pancreatic islets, α-toxin-permeabilization and most of the studies in paper II, III and IV. The microscope was equipped with a commercial TIRF illuminator (Nikon) and a 60x, 1.45-NA objective and a custom-built, experimental chamber holder thermostated to 37 °C. Lasers provided excitation at 457, 491, 515 and 561 nm. A filter wheel with integrated shutter and narrow band-pass filters were used for excitation control. Excitation and emission light were separated using a beamsplitter (ZT488/561rpc, Chroma Technology). Another filter wheel with interference filters (485 nm/25 nm half-bandwidth for CFP, 530 nm/50 nm half-bandwidth for GFP, 560 nm/40 nm half-bandwidth for YFP and 620 nm long-pass for mCherry) allowed rapid selection of emission wavelength. Fluorescence was detected with a back-illuminated electron-multiplying CCD camera.

The coverslips with attached cells were used as exchangeable bottoms of an open 200 or 50 µl chamber for the upright and inverted microscope setup, respectively, and superfused with buffer at a rate of 0.2-0.3 ml/min. A thin layer of immersion oil mediated optical coupling between the coverslip and the dove prism or objective. Both TIRF systems were controlled by MetaFluor software with image acquisition every 1 to 5 s. Changes of TIRF intensity over time was recorded from manually defined regions of interest corresponding to single cells using MetaFluor. The signal was normalized to the initial intensity after background subtraction.

Granule localization and exocytosis experiments were made using yet another objective-type TIRF microscope with a 100x, 1.45-NA objective. Excitation from lasers at 491 and 561 nm was controlled with an acousto-optical tunable filter. The emission light was chromatically divided onto separate areas of an electron-multiplying CCD camera using an dichroic image splitter with a cutoff at 565 nm and transmission at 525 nm/50 nm half-bandwidth for GFP and 600 nm/50 nm half-bandwidth for mCherry. The coverslips with attached cells were used as exchangeable bottoms of a 1.5 ml chamber thermostated to 32 °C. Exocytosis was triggered by local application of medium containing 75 mM K^+ through a pressurized glass electrode.

Confocal microscopy

The subcellular localization of the fluorescence-tagged or immunostained proteins were assessed using a spinning-disk confocal scanhead (Yokogawa CSU-10) attached to a Nikon microscope. Excitation light for GFP at 488 nm and Alexa Fluor-568 as well as mCherry at 561 nm were provided by lasers. An acousto-optic tunable filter was used for selection of excitation wavelength and to block the beam between image captures. Fluorescence from the cells was collected with a 60x, 1.4-NA oil immersion objective and detected through interference filter or glass filter with a back-illuminated EMCCD camera controlled by MetaFluor software.

Superfusion buffers and plasma membrane permeabilization

Most imaging experiments were performed in experimental buffer containing extracellular space-like salt concentrations (138 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 3 mM D-glucose, 25 mM HEPES and 0.1% BSA). In granule imaging experiments the cells were maintained in a similar solution (138 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, 3 mM D-glucose and 5 mM HEPES). To facilitate exocytosis the glucose concentration was increased to 10 mM, and the solution was supplemented with 2 µM forskolin and 200 µM of the K_{ATP} channel opener diazoxide. The fusion of granules was triggered by an increase of KCl to 75 mM (with equimolar reduction of NaCl). Buffers were adjusted to pH 7.40 by NaOH. In certain experiments MIN6 β-cells were permeabilized with Staphylococcus aureus α-toxin in an intracellular-like medium (140 mM KCl, 6 mM NaCl, 1 mM $MgCl_2$, 0.465 mM $CaCl_2$, 2 mM EGTA, 2 mM N-(2-hydroxyethyl)ethylenediamine-N,N′,N′-triacetic acid (HEDTA), 1 mM Mg-ATP, 0.25 mM Na-GTP, 100 µM IBMX, and 10 mM HEPES (pH adjusted to 7.00 with KOH)). The free concentrations of Ca^{2+} and Mg^{2+} were 0.1 µM and 1 mM.

Pull-down assay for Rap activity

The amounts of active Rap1 and total Rap1 in whole cell lysates were determined by Western blotting with a monoclonal anti-Rap1 antibody. MIN6 β-cells were lysed and the lysates were incubated with an excess of the antibody immobilized on beads to pull out active Rap1. A secondary antibody was applied, and a chemiluminescence reaction was carried out. Quantification of Rap1 protein was performed by densitometric analysis of Western blots.

Immunofluorescence

Immunostaining was used to analyze the subcellular distribution of the endogenous Epac2 protein. MIN6 cells grown on chamber slides were fixed and then incubated with an Epac2 mouse monoclonal antibody, followed by incubation with a secondary antibody. The chamber slides were finally mounted and the samples were imaged with a confocal microscope.

Results and Discussion

cAMP induces translocation of Epac2 from the cytoplasm to the plasma membrane (I)

Investigation of the subcellular localization by immunostaining for Epac2 in β-cells showed that the protein was mainly cytoplasmic in unstimulated cells but tended to localize to the plasma membrane after elevation of cAMP with the phosphodiesterase inhibitor IBMX. To study the kinetics of this translocation the cells were transfected with GFP-tagged Epac2. Like the endogenous protein, GFP-Epac2 distributed evenly in the cytoplasm under basal conditions and showed increased binding to the plasma membrane after stimulation with IBMX. The increased membrane fluorescence was much more obvious and easy to quantify with TIRF than confocal microscopy. The translocation of Epac2 to the plasma membrane was not secondary to PKA activation, since pharmacological PKA inhibition did not affect the IBMX response. The increased membrane binding was readily reversible and Epac2 dissociated from the plasma membrane within minutes after washout of the stimulus. The concentration-dependence of the translocation was determined in experiments with α-toxin-permeabilized cells where cAMP induced plasma-membrane association of Epac2 with half-maximal and maximal effects at 28 and 200 μM, respectively. In a previous study Epac2 was half-maximally activated by 20 μM cAMP [121], indicating that membrane translocation is closely related to Epac2 activation.

Cyclic translocation of Epac2 in glucose- and GLP-1-stimulated β-cells (I)

Physiological β-cell stimuli also induced plasma membrane translocation of Epac2. An increase in the glucose concentration from 3 to 11 mM triggered translocation of Epac2 after a few minutes delay. This delay is typical for the lag between glucose stimulation and the initiation of Ca^{2+} and cAMP signaling that drives insulin secretion [36,16]. The Epac2 translocation pattern varied between cells. Most cells responded with oscillations in membrane fluorescence with frequencies of $<0.3\ \mathrm{min}^{-1}$. Other cells showed faster oscillations, transient or stable Epac2 association to the plasma membrane. These findings indicate that the glucose-induced cAMP elevation in β-cells is sufficient to activate Epac2. In most glucose-stimulated cells, further elevation of cAMP by IBMX or GLP-1 triggered additional Epac2 translocation, which in the case of GLP-1 sometimes was associated with pronounced oscillations. GLP-1 triggered Epac2 translocation also in the presence of a substimulatory glucose concentration. The translocation response was not a phenomenon restricted to the MIN6 β-cell line, since glucose and cAMP-elevating agents were found to trigger Epac2 translocation also in primary cells within intact mouse and human pancreatic islets.

Epac2 accumulates at docked secretory granules (II)

To investigate how the Epac2 localization was related to insulin secretory granules, the cells were co-transfected with fluorescence-tagged Epac2 as well as NPY as marker for the granules. TIRF imaging selectively shows granules, which are present in the immediate vicinity of the plasma membrane. In unstimulated cells exposed to 3 mM glucose, fluorescence-labeled wildtype Epac2 distributed diffusely throughout the footprint area of the cell with slight preference for granules. After elevation of cAMP with forskolin, many cells showed clustering of Epac2 at the sites of docked granules. Quantitative analysis showed a 6-fold increase in fluorescence at granule sites in presence of forskolin. Clustering of Epac2 at granules was also induced by GLP-1 in both MIN6-cells and primary human islet cells.

A two-step mechanism underlies Epac2 recruitment to the plasma membrane and secretory granules (I, II, III)

Simultaneous TIRF recordings of Epac2 and the sub-plasma membrane cAMP concentration during glucose stimulation of the MIN6 β-cells showed that the Epac2 translocation was preceded by an increase in the cAMP concentration. Suppression of cAMP formation by adenylyl cyclase inhibitors reversed the glucose-induced Epac2 translocation, providing evidence for a critical role of cAMP in the process. Structural studies of Epac2 have shown that cAMP induces a large conformational change of the molecule [93] but the mechanism for Epac2 localization to the plasma membrane is unclear.

To determine the importance of different domains in Epac2 for the translocation and granule accumulation processes, we used various fluorescent-protein-tagged mutants, which were co-expressed with differently tagged wildtype Epac2 molecules to enable direct comparison of the translocation responses in the same cell. Control experiments showed nearly identical translocation of co-expressed GFP- and mCherry-tagged wildtype Epac2, indicating that the translocation responses can be quantitatively compared between different Epac2 constructs.

Confocal imaging under basal conditions revealed that most Epac2 mutants were like wild-type distributed in the cytoplasm. Only the Epac2 mutants lacking the entire CNB region or the DEP domain showed increased membrane association in the basal state. TIRF imaging disclosed that IBMX- and GLP-1-induced translocation was strongly impaired in mutants with reduced cAMP binding to the high affinity site, but unaffected or even enhanced after deletion or mutation of the low-affinity cAMP-binding site. Similar observations were made in glucose-stimulated cells, but elimination of cAMP binding had a less pronounced effect on the translocation. Experiments in permeabilized cells showed left-shifted cAMP-dependence, but reduced maximal translocation in the CNB1-deficient Epac2 mutant. The increased translocation after deletion of the CNB1 domain may reflect increased accessibility of cAMP to the high-affinity CNB2 domain, since the crystal structure indicates that the two domains occlude each other [90].

Analyzing Epac2 clustering at secretory granules it was found that cAMP elevation failed to induce clustering of the CNB1 mutants, indicating that although this domain is not involved in plasma membrane translocation, it is responsible for the association of Epac2 with secretory granules. Consistent with this conclusion, expression of the isolated CNB1 domain demonstrated significant co-localization with vesicles. GLP-1 stimulation tended to reduce this clustering, possibly because of competition from endogenous Epac2. The role of the CNB1 domain for association with the exocytosis machinery was also supported by the observation that Epac1, which lacks CNB1 domain, failed to cluster at granule docking sites, although the protein showed pronounced translocation to the plasma membrane in response to cAMP. Direct comparison of the cAMP-induced translocation of co-expressed Epac1 and Epac2 in permeabilized cells showed that the dose-dependence of cAMP-induced translocation for Epac1 was more similar to the CNB1-deficient mutant than to wild-type Epac2. Given the importance of the CNB1 domain for the interaction with the exocytosis machinery it is perhaps not surprising that the domain is only present in the Epac2A splice variant, which is the dominating Epac2 version in secretory cells like neurons and β-cells [79].

DEP domains mediate membrane binding in many proteins, including Epac1 [122], but the role of the DEP domain in Epac2 is unclear. The translocation of Epac2 lacking the DEP domain was apparently reduced in intact cells stimulated with IBMX or GLP-1, as well as in permeabilized cells exposed to cAMP. Since the pre-stimulatory signal was used as reference, the small response amplitude may reflect the increased association of the DEP-deficient Epac2 mutant with the plasma membrane and granule sites under basal conditions. In contrast to the response to cAMP-elevating agents, that to glucose was unaffected by DEP deletion.

Previous studies have indicated that Epac2 interacts with Ras at the plasma membrane via its RA domain [89,123]. To determine the role of this domain for Epac2 translocation we used an Epac2 mutant lacking the RA domain or having a point mutation in the critical Ras binding site (K684E). After IBMX- or GLP-1-induced increases in the cAMP concentration, the Epac2-K684E mutant showed very weak translocation to the plasma membrane. The translocation response was impaired also in permeabilized cells exposed to cAMP. In glucose-stimulated cells, Epac2-K684E translocation was suppressed and did not show oscillations like the wildtype protein. Consistent with the involvement of Ras binding, the glucose-induced translocation of wildtype Epac2 was impaired when overexpressing a dominant-negative Ras mutant.

These observations point to a essential role of cAMP binding to the high-affinity binding site in CNB2 to induce translocation, although additional mechanisms may contribute to membrane translocation in glucose-stimulated cells. The membrane recruitment depends strongly on interaction with Ras via the Epac2 RA domain. In contrast to the previously proposed role of the CNB1 domain for membrane targeting of Epac2 [79], this domain seemed to promote membrane binding only at extremely high cAMP concentrations, which may not be reached under physiological conditions. The importance of this domain is instead to target Epac2 to secretory granule sites, and the present work indicates a two-step recruitment process whereby Epac2 first translocates to the plasma membrane and then becomes associated with the granule docking sites (Fig. 4). The role of the DEP domain remains unclear. Although the present data show that it is not required for membrane binding of Epac2, it seems to influence the localization mediated by other parts of the protein.

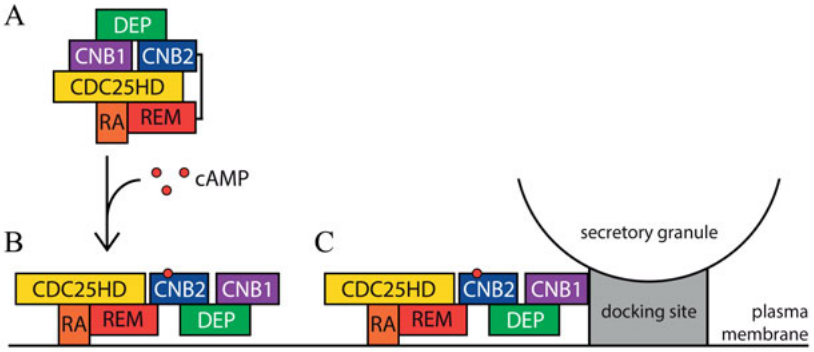

Figure 4: *Model for the two-step mechanism underlying recruitment of Epac2 to secretory granule docking sites at the plasma membrane* (A) Inactive Epac2 protein located in the β-cell cytoplasm. (B) cAMP binding to CNB2 induces a conformational change. In the activated conformation, the RA domain interacts with the plasma membrane. (C) The plasma membrane localization allows CNB1 to interact with proteins associated with vesicle docking sites where Epac2 promotes granule priming and exocytosis.

Dual effects of Ca^{2+} on Epac2 translocation (I, III)

Since glucose promoted membrane recruitment of Epac2 and glucose stimulation of β-cells involves increase and pronounced oscillations of the cytoplasmic Ca^{2+} concentration [5], it was of interest to investigate the relationship between Ca^{2+} and Epac2 localization. Simultaneous recordings of the sub-plasma membrane Ca^{2+} concentration and Epac2 membrane binding showed that the glucose-induced Epac2 translocation was preceded by an increase in the Ca^{2+} concentration and that removal of extracellular Ca^{2+} immediately reduced the plasma membrane association, indicating that Ca^{2+} influences the translocation of Epac2.

cAMP-dependent Epac2 translocation was reduced and slower under conditions preventing increase of the cytoplasmic Ca^{2+} concentration. This effect was not due to altered cAMP production and/or degradation via Ca^{2+}-sensitive adenylyl cyclases and phosphodiesterases, because the translocation was reduced also in response to membrane permeable 8-Br-cAMP. These results indicate that Ca^{2+} has a permissive role for cAMP-induced Epac2 translocation to the plasma membrane.

Increase of intracellular Ca^{2+} by depolarization with a high K^{+} concentration in the presence of 3 mM glucose induced an increase of Epac2 plasma membrane binding in most cells. If the cells were already exposed to cAMP-elevating agents some responded to the depolarization-evoked Ca^{2+} elevation with increased Epac2 binding to the plasma membrane, whereas Ca^{2+} elevation in other cells induced dissociation of Epac2 from the membrane. A correlation was found between the amplitude of the cAMP-induced translocation response and the effect of the subsequent depolarization with a stronger inhibitory effect of Ca^{2+} the more Epac2 was localized to the plasma membrane. The negative effect of Ca^{2+} on Epac2 membrane localization could not be ascribed to lowering of the cAMP concentration that may occur by activation of Ca^{2+}-sensitive phosphodiesterases or inhibition of Ca^{2+}-sensitive adenylyl cyclases present in β-cells [58]. It was consequently observed also in permeabilized cells when the intracellular cAMP concentration was strictly controlled. The conclusion from these observations is that Ca^{2+} has dual effects on Epac2 plasma membrane binding, and that the response is influenced by the presence of cAMP.

Ca^{2+}-mediated Ras activation triggers translocation (I)

As the Ras GTPase is involved in Epac2 membrane recruitment and Ca^{2+} is known to regulate Ras activity [124-126], the role of Ras in Ca^{2+}-stimulated Epac2 translocation was investigated in experiments where Ras activity at the plasma membrane was monitored using TIRF microscopy and a biosensor consisting of the Ras-binding domain from Raf1 fused to a fluorescent protein ($Raf1_{RBD}$). Increase of the glucose concentration induced translocation of $Raf1_{RBD}$ to the plasma membrane, indicating increased Ras activity in this compartment. Simultaneous measurements revealed that oscillations of Epac2 translocation induced by glucose were paralleled by oscillations of $Raf1_{RBD}$. These effects on Ras activity are probably driven by the glucose-induced changes of the cytoplasmic Ca^{2+} concentration. Accordingly, increases in the Ca^{2+} concentration generated by repeated K^+ depolarizations consistently induced synchronized membrane recruitment of $Raf1_{RBD}$ and Epac2 that depended on the presence of Ca^{2+} in the extracellular medium. When studying Epac2 in permeabilized cells, Ca^{2+} did not influence the translocation response unless GTP was present in the buffer, supporting the involvement of a G protein in the process. These findings support the conclusion that Ca^{2+} controls Epac2 localization by changes in Ras activity. The effect is most likely mediated via Ca^{2+}-sensitive GEFs, such as GRF1, which is expressed in β-cells [124].

Epac2 membrane binding requires PIP_2 (III)

The reason for the negative effect of Ca^{2+} on membrane binding of Epac2 was next investigated. Since voltage-dependent Ca^{2+} influx is known to lower the membrane PIP_2 levels via activation of PLC [127-129], and PIP_2 is involved in the anchoring of many proteins, including K-Ras, to the plasma membrane [130], it was hypothesized that this lipid somehow is involved in the regulation of Epac2 localization. This idea was tested using an optogenetic system, which allows light-induced recruitment of an inositol 5-phosphatase domain to the plasma membrane [119]. In MIN6 β-cells expressing the constructs together with a PIP_2 biosensor, exposure to blue light induced rapid loss of PIP_2 from the plasma membrane.

In cells co-expressing Epac2 and the light-controlled phosphatase, blue light strongly counteracted Epac2 translocation stimulated by IBMX and forskolin. Control experiments showed that this effect was not due to a reduction of the cAMP concentration. It is unclear how this effect of PIP_2 is mediated. The lipid might interact directly with Epac2, although not with the DEP domain, as also the DEP-deficient Epac2 mutant dissociated from the membrane in response to PIP_2 depletion. In this respect, Epac2 differs from Epac1, whose membrane binding relies on an interaction between the DEP domain and phosphatidic acid. It is possible that Epac2 binding to PIP_2 occurs indirectly through interactions with other PIP_2 effectors. An obvious candidate is K-Ras, which in addition to a posttranslational C-terminal prenylation, contains a polybasic motif that mediates important interaction with negatively charged phospholipids, including PIP_2 [130]. Reduced association of Ras with the plasma membrane can be expected to decrease that of Epac2. However, in control experiments, PIP_2 depletion was without effect on the membrane binding of a fluorescent protein fused to the polybasic region and the CAAX motif from K-Ras. If Ras activity is influenced by PIP_2 by some other means is unknown. These findings reveal a novel potential regulatory mechanism on Epac2-dependent processes at the plasma membrane. PIP_2 is important for exocytosis [43] and it is possible that the lipid contributes to the clustering of Epac2 at the secretory granule docking sites.

Epac2 translocation activates Rap1 at the plasma membrane (I)

To investigate if Epac2 translocation was associated with activation of its major effector at the plasma membrane, activity was monitored using a fluorescent protein-tagged Rap-binding domain from RalGDS [111]. When Epac was activated with 007-AM or a combination of forskolin and IBMX, there was stable translocation of the Rap reporter, indicating activation of Rap GTPases at the plasma membrane. Subsequent depolarization with K^+ enhanced the Rap activity. These results with the fluorescent reporter were confirmed in a traditional Rap-GTP pull-down assay. Increase of the glucose concentration induced oscillatory translocation of the Rap reporter in both MIN6 and primary mouse β-cells, indicating periodic plasma membrane Rap activity. Consistent with the involvement of Ras in the glucose-induced Epac2 activation process, expression of a dominant-negative Ras mutant reduced the glucose-induced Rap activity.

Binding of Epac2 to the exocytosis machinery promotes granule priming (I, II)

An important question is whether Epac2 recruitment to the plasma membrane and secretory granules influences insulin secretion. To clarify this issue exocytosis of granules containing a fluorescent protein marker was visualized with TIRF microscopy. The cells were depolarized with a high K^+ concentration and exocytotic events, seen as rapid disappearance of the granule marker fluorescence, were detected immediately after stimulation. The granule-associated Epac2 signal was compared between granules that underwent exocytosis and those that did not and this analysis showed significantly higher amounts of Epac2 associated with exocytosing granules compared to those that were only docked, in which the Epac2 signal was indistinguishable from background. This finding indicates that recruitment of Epac2 to the plasma membrane correlates with granule priming, which is consistent with a role of Epac2 in this process. It is not clear how Epac2 promotes priming, but it is probably related to its interaction with RIM2α [104] or other components of the secretory machinery, such as the SUR1 subunit of the K_{ATP} channel [73], the granular sulphonylurea receptor [101] voltage-dependent Ca^{2+} channels [102], the active zone protein piccolo [102] or SNAP25 [103]. An interesting possibility is that Epac2 promotes granule exocytosis by stimulating vesicular uptake of glutamate [131].

The Rap proteins have also been implicated in secretory granule exocytosis [85]. To evaluate their involvement in insulin secretion, the kinetics of secretion from individual MIN6 β-cells was monitored with a fluorescent translocation biosensor in an assay based on detection of the phospholipid PIP_3, formed after autocrine activation of insulin receptors [116,16]. Control cells expressing the biosensor responded to glucose with oscillations in fluorescence intensity, which reflect pulsatile insulin secretion, and K^+ depolarization induced a prompt increase of the signal. The magnitudes of the translocation responses were significantly reduced in cells expressing a dominant-negative mutant of Rap1. These findings indicate that glucose stimulation of insulin secretion from β-cells involves activation and recruitment of Epac2 to the plasma membrane where the protein activates Rap1 and other potential effectors that promote secretory granule priming and exocytosis.

Sulfonylureas trigger translocation of Epac2 to the plasma membrane (IV)

A suggestion that Epac2 is a direct target of sulfonylurea action [94] has been debated [98]. In an attempt to clarify the potential link between Epac2 and sulfonylureas, TIRF imaging was used to monitor Epac2 membrane binding in MIN6 β-cells during exposure to 1 mM of the sulfonylurea tolbutamide in the presence of a sub-stimulatory concentration of glucose. In approximately half of the cells, tolbutamide triggered Epac2 translocation, indicating activation of the protein, while in the remaining cells, there was no effect or even slight reduction of membrane binding. In most of the responding cells, there were periodic increases and decreases in Epac2 membrane binding reminiscent of the oscillations observed in glucose-stimulated β-cells. Similar stimulation of Epac2 translocation was observed with the more potent second generation sulfonylureas glibenclamide and gliclazide, although gliclazide is not supposed to directly interact with Epac2 [94,96].

Sulfonylurea-induced Epac2 translocation depends on voltage-dependent Ca^{2+}-influx (IV)

Sulfonylureas depolarize β-cells by closing K_{ATP} channels in the plasma membrane, which results in L-type voltage-dependent Ca^{2+} influx. Parallel measurements of Epac2 membrane binding and the sub-plasma membrane Ca^{2+} concentration showed that distinct, periodic increases of Ca^{2+} induced by tolbutamide, glibenclamide or gliclazide were followed by periods of increased Epac2 binding to the plasma membrane. Inhibition of L-type Ca^{2+} channels with methoxyverapamil (D600) prevented the Ca^{2+} increases and reduced the Epac2 membrane binding. There was no effect of tolbutamide on the basal levels of cAMP. Together these, observations support the conclusion that sulfonylureas activate Epac2 indirectly via Ca^{2+} rather than via a direct action on the protein.

Dual effects of tolbutamide on Epac2 membrane binding in the presence of cAMP (IV)

Sulfonylureas have been proposed to activate Epac2 cooperatively with cAMP [96]. The effect of tolbutamide on Epac2 membrane binding was therefore evaluated in cells after forskolin-induced elevation of cAMP. As elevations of cAMP sometimes induces Ca^{2+} signaling in MIN6 β-cells we tested the potential interaction between cAMP and sulfonylurea in primary β-cells within intact mouse pancreatic islets. Forskolin at 1 μM always increased Epac2 binding to the plasma membrane but subsequent addition of tolbutamide induced variable responses. In about half of the cells, there was increased Epac2 binding to the plasma membrane, about 30% showed decreased binding and there was no clear effect of tolbutamide in the remaining cells. In similar experiments with a tenfold higher concentration of forskolin to increase cAMP further, tolbutamide induced Epac2 dissociation in most of the cells. These results are strongly reminiscent of the dual effects of Ca^{2+} on Epac2 membrane binding described above and probably reflect similar stimulatory effects of Ca^{2+} on Ras activity and reduction of the concentration of PIP_2.

The effect of tolbutamide in the presence of cAMP was further investigated in permeabilized MIN6 β-cells. This approach allows clamping of the concentrations of cAMP and Ca^{2+} and thereby circumvents the potential confounding effects of the complicated relationship between Ca^{2+} and Epac2. cAMP induced rapid translocation of Epac2 to the plasma membrane in the permeabilized cells but subsequent addition of tolbutamide was without effect. These findings lend further support for the conclusion that the effects of sulfonylurea are indirect and mediated by changes of the cytoplasmic Ca^{2+} concentration.

Sulfonylureas activate Rap in a Ca^{2+}-dependent manner (IV)

Although Epac2 translocation to the plasma membrane is an important step in the activation process it is possible that a direct effect of sulfonylureas is overlooked if the drugs activate Epac2 that is already present at the plasma membrane. Most sulfonylureas are not readily plasma membrane permeable [132] and it is therefore conceivable that their action is restricted to membrane-associated targets. The effects of tolbutamide and gliclazide were therefore evaluated on plasma membrane Rap activity in MIN6 β-cells expressing the fluorescent Rap reporter [111]. Consistent with their effects on Epac2 translocation, we found that not only tolbutamide, but also gliclazide, increased Rap activity at the plasma membrane, and that this action was reversed by inhibition of L-type voltage-dependent Ca^{2+} channels. These results indicate that sulfonylureas indeed stimulate Epac2 activity and downstream Rap signaling, but that this effect is caused by the concomitant increases of the cytoplasmic Ca^{2+} concentration. There is consequently strong evidence in favour of the view that the interaction between sulfonylureas and Epac2 is mediated indirectly. More studies are required to clarify the relevance of the interaction between Epac2 and SUR1 [73] for the effects of sufonylureas. SUR1 not only functions as a subunit of the K_{ATP} channel, but is also involved in regulating cAMP-dependent, protein-kinase-A-independent, granule priming [101].

Conclusions

1. The subcellular localization of Epac2 is dynamically controlled by cAMP, Ca^{2+}-mediated Ras activity and PIP_2, which promote translocation of the protein to the plasma membrane. Physiological stimulation with glucose or GLP-1 induces oscillatory Epac2 translocation between the cytoplasm and the plasma membrane in insulin-secreting β-cells.

2. The spatial redistribution of Epac2 involves multiple domains in both the regulatory and catalytic regions of the protein. cAMP binding to the high-affinity domain, as well as an intact RA domain, are crucial for the plasma membrane interaction. Neither the DEP domain, nor the low-affinity CNB1 domain is required for membrane binding, but the CNB1 domain mediates targeting of Epac2 to secretory granule docking sites.

3. Epac2 clusters at secretory vesicle docking sites in a process correlating with priming of insulin granules for exocytosis.

4. The translocation of Epac2 is associated with increased Rap GTPase activity at the plasma membrane and this process contributes to amplification of insulin secretion.

5. Sulfonylureas induce binding of Epac2 to the plasma membrane in β-cells irrespective of their supposed ability to directly interact with Epac2. This effect is indirect and mediated by their depolarizing action on β-cells, which leads to increased cytoplasmic Ca^{2+} concentration.

Summary in Swedish

Glukoskoncentrationen i blodet hålls inom ett ganska snävt intervall, trots stora variationer i energiintag och förbrukning p.g.a. matintag, motion eller nattens vila och fasta. Eftersom glukos är ett oumbärligt näringsämne för hjärnan, är mycket låga blodsockernivåer akut livshotande. Höga blodglukoskoncentrationer, å andra sidan, är ingen akut fara, men det har långsiktiga negativa effekter på många vävnader och organ. Kontroll av blodsockret är därför mycket viktigt och denna funktion utövas genom ett samspel mellan flera vävnader och organ, inklusive bukspottkörteln, levern, hjärnan, muskler och fettväv.

Bukspottkörteln är en utmärkt regulator av glukosbalansen genom att producera och frigöra blodglukosreglerande hormoner. Majoriteten av körteln (99%) är exokrin och producerar magsafter och endast en mycket liten del (1%) är endokrin och frigör hormoner till blodet. Den endokrina delen består av grupper på upp till några tusen celler som kallas Langerhanska öar. Dessa cellöar innehåller fem olika endokrina celltyper: α-, β-, δ-, ε- och PP-celler. De flesta av cellerna är β-celler, som producerar och frisätter insulin som svar på ökande blodglukosnivåer, andra näringsämnen, hormoner och neurala faktorer. Insulin sänker blodsockret genom att främja glykogenbildning och undertrycka glykogenolysen och glukoneogenesen i levern samt genom att främja glukosupptag och lagring i muskler och fettvävnad. Avsaknad av eller störd funktion hos β-celler är orsaken till de flesta former av diabetes, som kännetecknas av bristande insulinutsöndring med resulterande hyperglykemi.

När blodglukos ökar efter en måltid transporteras sockret in i β-cellen där metabolism genererar ATP. Detta leder till stängning av ATP-känsliga kaliumkanaler i plasmamembranet varvid membranet depolariseras och spänningskänsliga kalciumkanler öppnas så att jonen kan flöda in i cellen. Det glukosinducerade kalciumsvaret karakteriseras av periodiska variationer, så kallade oscillationer, vilka genererar pulserande insulinfrisättning hos friska individer.

Glukos stimulerar även insulinfrisättningen genom att producera metabola faktorer, som förstärker den frisättning som induceras av kalcium. Denna förstärkande mekanism är inte helt klarlagd och flera möjliga faktorer har diskuterats. En sådan är cAMP som bildas från ATP och som är avgörande för en optimal insulinfrisättning. Det är därför inte förvånande att cAMP oscillerar i fas med kalcium.

Den ökade cAMP-koncentrationen aktiverar proteinkinas A och det G-protein reglerande proteinet Epac. Hur Epac förmedlar sin effekt på insulinfrisättningen är inte helt klarlagt. I denna avhandling fokuserar jag på Epac. Detta sker genom att mestadels använda totalreflektionsmikroskopi, en teknik som möjliggör att studera vad som händer vid cellers plasmamembran.

I avhandlingens första arbeten visas att ökningar i cAMP-koncentrationen fick Epac2 att translokera från cytoplasman till plasmamembranet i β-cellerna. Glukosinducerade oscillationer av cAMP orsakade oscillationer av Epac2-translokering. Translokeringen kunde dessutom förstärkas genom ökning av cytoplasmiskt kalcium. Försök med mutanter i Epac2 visade att domäner som binder cAMP med hög affinitet och som associerar till G-proteinet Ras var avgörande för Epac2s translokering. Epac2 aktiverar Rap GTPas och vi kunde visa att oscillationer av Epac2 åtföljdes av svängningar av Rap aktivitet vid β-cellens plasmamembran. Däremot krävdes varken den s.k. DEP domänen, eller en domän som binder cAMP med låg affinitet för membraninteraktionen. Det visade sig även att Epac2 företrädesvis ansamlades där insulingranula var bundna till plasmamembranet. Denna ackumulering berodde på närvaro av den cAMP-bindande Epac2-domänen med låg affinitet för nukleotiden och gjorde granula mer benägna att frisättas genom exocytos vid membrandepolarisering. Vår slutsats är att Epac2 kontrollerar insulinfrisättning genom att translokera till platser för exocytos vid plasmamembranet. Processen omfattar två distinkta lokaliseringsmekanismer, en för rekrytering av Epac2 till cellmembranet och en annan för associering med exocytosmaskineriet.

I avsaknad av andra stimuli, kan en depolarisation av plasmamembranet och resulterande höjning av kalcium inducera Epac2-translokering till plasmamembranet, medan kalciumökningar i celler där Epac2 translokeringen redan stimulerats genom cAMP-höjning ofta resulterar i minskad Epac2-interaktion med plasmamembranet. Samma fenomen observerades även i permeabiliserade celler som utsattes för en konstant cAMP koncentration men olika kalciumkoncentrationer. Eftersom ökningar av intracellulärt kalcium leder till minskade nivåer av membranfosfolipiden PIP_2 testade vi om den påverkade membranassociationen av Epac2 genom att bryta ned PIP_2 vid plasmamembranet. Med hjälp av optogenetisk teknik kunde PIP_2 nedbrytning induceras med belysning. Detta utlöste en uttalad dissociation av Epac2 från plasmamembranet i celler som redan exponerats för cAMP höjande stimuli. Detta tyder på att den subcellulära lokaliseringen av Epac2 styrs av ett komplext samspel mellan cAMP, kalcium och PIP_2. PIP_2 är alltså en faktor som kan påverka Epac2-reglerade processer vid plasmamembranet.

Den antidiabetiska läkemedelsgruppen sulfonylurea ökar insulinutsöndring från β-celler. Detta genom att sulfonylurea binder till och inhiberar ATP-känsliga kaliumkanaler, vilket leder till depolarisation av plasmamembranet, kalciuminflöde och exocytos av insulingranula. Det har nyligen föreslagit att sulfonylurea också kan binda direkt till och aktivera Epac2, men det är kontroversiellt. Vi undersökte därför kopplingen mellan sulfonylurea och Epac2. Därvid använde vi oss av tre olika sulfonylureasubstanser, tolbutamid, glibenklamid och gliclazid. Alla stimulerade rekryteringen av Epac2 till plasmamembranet, trots att gliclazid inte binder direkt till Epac2. Den ökade membranbindning av Epac2 korrelerade med ökningar av den cytoplasmiska kalciumkoncentration och hämmades då av inflödet av kalcium blockerades. När Epac2 stimuleras av cAMP, inducerar tolbutamid ytterligare bindning i vissa celler, men främjar dissociation i andra, detta speglar de dubbla effekterna av kalcium på Epac2 translokering. Mätningar av Rap-aktivitet vid plasmamembranet visade att tolbutamid och gliclazid båda aktiverade Rap på ett kalciumberoende sätt. Vi drar här därför slutsatsen att sulfonylurea stimulerar aktiveringen av Epac2 till plasmamembranet på ett indirekt sätt genom att depolarisera β-cellens plasmamembran och inducera en ökning av den cytoplasmiska kalciumkoncentrationen.

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