Integration of ATP, cAMP, and Ca^{2+} Signals in Insulin Granule Exocytosis

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Intracellular ATP, cAMP, and Ca^{2+} are major signals involved in the regulation of insulin secretion in the pancreatic β-cell. We recently found that the ATP-sensitive K^+ channel (K_{ATP} channel) as an ATP sensor, cAMP-GEFII as a cAMP sensor, Piccolo as a Ca^{2+} sensor, and L-type voltage-dependent Ca^{2+} channel (VDCC) can interact with each other. In the present study, we examined the effects of cAMP and ATP on the interaction of cAMP-GEFII and sulfonylurea receptor-1 (SUR1). Interaction of cAMP-GEFII with SUR1 was inhibited by the cAMP analog 8-bromo-cAMP but not by ATP, and the inhibition by 8-bromo-cAMP persisted in the presence of ATP. In addition, SUR1, cAMP-GEFII, and Piccolo could form a complex. Piccolo also interacted with the $\alpha_1$1.2 subunit of VDCC in a Ca^{2+}-independent manner. These data suggest that the interactions of the K_{ATP} channel, cAMP-GEFII, Piccolo, and L-type VDCC are regulated by intracellular signals such as cAMP and Ca^{2+} and that the ATP, cAMP, and Ca^{2+} signals are integrated at a specialized region of pancreatic β-cells. *Diabetes* 53 (Suppl. 3):S59–S62, 2004

Stimulus-secretion coupling is a crucial event in secretory cells, including neuronal, neuroendocrine, endocrine, and exocrine cells (1). Cells in which regulated exocytosis occurs share several molecules that constitute the exocytotic machinery, including SNARE proteins (VAMP/synaptobrevin, SNAP, and syntaxin), synaptotagmins, and Rab proteins (2,3). An increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is the primary signal in most secretory cells. In neurons, the elevation of $[Ca^{2+}]_i$ results from Ca^{2+} influx owing to opening of the voltage-dependent Ca^{2+} channels (VDCCs) localized in the active zone, a specialized region where synaptic vesicles dock and fuse (4,5). CAZ (*c*ytoskeletal matrix associated with the *a*ctive *z*one) proteins have been suggested to organize the site of neurotransmitter release. These include Piccolo/Aczonin (6,7), Bassoon (8), Rim1 (9), Munc13-1 (10), and CAST/ERC (11,12). Piccolo/Aczonin is a 500-kDa protein with zinc fingers, a PDZ (PSD-95, Dlg, and ZO-1) domain, and two C_2 domains. Rim1, which is structurally related to Piccolo/Aczonin, is a 180-kDa protein that interacts with Rab3 (9). The pancreatic β-cell is a typical endocrine cell in which exocytosis of insulin-containing vesicles is regulated by a variety of intracellular signals. ATP, cAMP, Ca^{2+}, and diacylglycerol are the major intracellular signals involved in the regulation of insulin secretion (13).

We recently found that cAMP-GEFII/Epac2 (hereafter cAMP-GEFII) (14–16), acting as a cAMP sensor, interacts specifically with sulfonylurea receptor-1 (SUR1) through nucleotide-binding fold (NBF)-1 of SUR1 (16). We also found that cAMP-GEFII mediates cAMP-dependent, protein kinase A (PKA)-independent insulin secretion, and that this requires interaction with both Rim2 and Piccolo (16–18). Piccolo forms a homodimer or a heterodimer with Rim2 in a Ca^{2+}-dependent manner, and Piccolo rather than Rim2 may function as the Ca^{2+} sensor (18). In addition, Rim2 and Piccolo bind directly to $\alpha_1$1.2-subunit of VDCCs (19). These findings show that the ATP, cAMP, and Ca^{2+} sensors interact with each other.

In the present study, we investigated regulation of the interaction of the ATP-sensitive K^+ channel (K_{ATP} channel) and cAMP-GEFII. We also show direct interaction of SUR1, cAMP-GEFII, and Piccolo.

RESEARCH DESIGN AND METHODS

Recombinant fusion proteins. SUR1 (amino acid residues 598–1,003), SUR1 (598–762), Piccolo (4,505–4,758), Piccolo-C_2A (4,704–5,010), and Piccolo-C_2B (4,955–5,165) were expressed as a glutathione S-transferase (GST)-fused protein in BL21. The fusion proteins were purified by affinity chromatography with glutathione-resin (Amersham Biosciences). SUR1 (598–1,003) was also expressed as a maltose-binding protein (MBP)-fused protein in BL21. These fusion proteins were purified by affinity chromatography with amylose-resin (New England BioLabs). Fragment containing $\alpha_1$1.2-subunit (745–892) was subcloned in pGBKT7 vector (Clontech Laboratories, Palo Alto, CA) as a Myc-tagged protein.

Pull-down assay. Mouse insulin-secreting MIN6 cells were sonicated in binding buffer (20 mmol/l HEPES, pH 7.4, 150 mmol/l NaCl, 1 mmol/l EGTA, 5 mmol/l $MgCl_2$, and 0.5% NP-40). The cellular lysates were incubated with 0.5 μg GST-fused protein immobilized on glutathione-resin for 90 min at 4°C. The washed complexes were separated by SDS-PAGE and subjected to immunoblot analysis with anti-cAMP-GEFII antibody (16). COS-1 cells were transfected with pCMV-FLAG-mouse cAMP-GEFII using LipofectAMINE PLUS (Invitrogen, Carlsbad, CA). The transfected cells were sonicated in binding buffer. The cellular lysates were incubated with MBP-SUR1 (598–1,003) immobilized on amylose-resin for 90 min at 4°C. The washed complex was incubated with GST-Piccolo (4,505–4,758) for 2 h at 4°C. The sample was

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$[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CAZ, cytoskeletal matrix associated with the active zone; Epac, exchange protein directly activated by cAMP; GEF, guanine nucleotide exchange factor; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; GST, glutathione S-transferase; K_{ATP} channel, ATP-sensitive K^+ channel; MBP, maltose-binding protein; NBF, nucleotide-binding fold; PKA, protein kinase A; SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SUR, sulfonylurea receptor; VAMP, vesicle-associated membrane protein; VDCC, voltage-dependent Ca^{2+} channel.

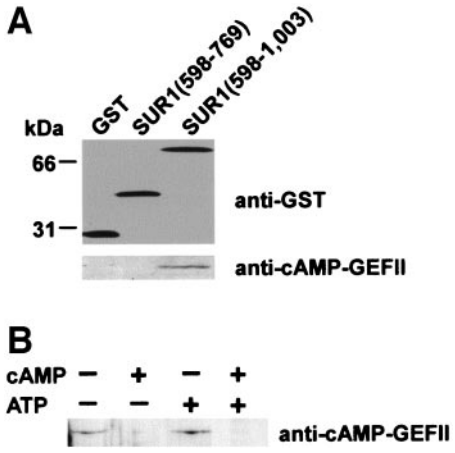

FIG. 1. Binding properties of cAMP-GEFII and SUR1. *A*: Binding of cAMP-GEFII to SUR1. The lysates of MIN6 cells were incubated with GST alone, GST-SUR1 (598–769), or GST-SUR1 (598–1,003). The samples were analyzed by immunoblotting with anti-GST antibody and anti-cAMP-GEFII antibody. The data are representative of two experiments. *B*: Effects of 8-bromo-cAMP and ATP on binding of cAMP-GEFII to SUR1. The lysates of MIN6 cells were incubated with MBP-SUR1 (598–1,003) in the presence of 1 mmol/l 8-bromo-cAMP and/or 1 mmol/l ATP. The samples were analyzed by immunoblotting with anti-cAMP-GEFII antibody. The data are representative of three experiments.

separated by SDS-PAGE and subjected to immunoblot analysis with anti-GST antibody (Amersham Biosciences) and anti-FLAG M2 antibody (Sigma).

Interaction of GST-fused Piccolo-C_2A (4,704–5,010) or Piccolo-C_2B (4,955–5,165) with fragment of $\alpha_1$1.2-subunit (745–892) was performed as described previously (19).

RESULTS

We previously identified the cAMP-binding protein cAMP-GEFII by a yeast two-hybrid screen of MIN6 cDNA with a partial SUR1 (amino acid residues 598–1,003) as bait (16). cAMP-GEFII specifically binds to NBF-1 of SUR1 (19). To determine if the binding of cAMP-GEFII to NBF-1 of SUR1 requires both the Walker A and the Walker B motifs, the lysates of MIN6 cells were incubated with GST-SUR1 (598–769) lacking Walker A or SUR1 (598–1,003) containing NBF-1 immobilized on glutathione-resin. As shown in Fig. 1*A*, SUR1 (598–769) did not bind to cAMP-GEFII, indicating that cAMP-GEFII binds to intact NBF-1 of SUR1.

We then examined the effect of ATP on binding of cAMP-GEFII to SUR1. The lysates of MIN6 cells were incubated with GST-SUR1 (598–1,003) in the presence of 1 mmol/l 8-bromo-cAMP and/or 1 mmol/l ATP. Interaction of cAMP-GEFII and SUR1 was inhibited by the cAMP analog 8-bromo-cAMP, but was not inhibited by ATP (Fig. 1*B*). In addition, the interaction was inhibited by the presence of both 8-bromo-cAMP and ATP. These results suggest that the interaction of cAMP-GEFII and NBF-1 of SUR1 is not regulated by binding of ATP to NBF-1 but by binding of cAMP to cAMP-GEFII.

Because cAMP-GEFII interacts with the Ca^{2+} sensor Piccolo through its PDZ domain (18), we examined whether Piccolo binds to a complex of SUR1 and cAMP-GEFII. FLAG-cAMP-GEFII, expressed in COS-1 cells, was incubated with MBP-SUR1 (598–1,003) immobilized on amylose-resin, followed by incubation with GST-Piccolo (4,505–4,758) containing the PDZ domain. The results showed that Piccolo binds to the SUR1·cAMP-GEFII complex (Fig. 2*A*).

Two C_2 domains in Piccolo interact with the cytoplasmic loop of $\alpha_1$1.2-subunit of L-type VDCC in the absence of Ca^{2+} (19). We also examined whether these C_2 domains interact with $\alpha_1$1.2-subunit in the presence of Ca^{2+}. The C_2 domains of Piccolo bind directly to $\alpha_1$1.2-subunit of VDCC (Fig. 2*B*) in a Ca^{2+}-independent manner.

DISCUSSION

The K_{ATP} channel in pancreatic β-cells plays a critical role in the regulation of glucose-induced and sulfonylurea-induced insulin secretion (20,21). The β-cell K_{ATP} channel is a hetero-octamer composed of pore-forming Kir6.2 subunits and regulatory SUR1 subunits (20,21). SUR1 has two intracellular nucleotide-binding folds (NBF-1 and NBF-2), each containing a Walker A and a Walker B motif. We recently established that the cAMP-binding protein cAMP-GEFII specifically interacts with NBF-1 of SUR1 (16,19). We have now found that the interaction requires both the Walker A and Walker B motifs. ATP has been shown to bind to NBF-1 of SUR1 (22), but the binding of ATP to NBF-1 did not affect interaction of SUR1 and cAMP-GEFII. Because cAMP-GEFII does not affect β-cell K_{ATP} channel activity under the conditions used (19), these findings suggest that SUR1 may function as a scaffold protein in addition to its role as the regulatory subunit of the K_{ATP} channel.

Interaction of cAMP-GEFII and SUR1 is decreased by a high cAMP concentration (19). Because binding of cAMP to cAMP-GEFII induces a conformational change of cAMP-GEFII (23), cAMP-GEFII might dissociate from the complex of SUR1 and cAMP-GEFII upon cAMP stimulation. Accordingly, rather than being activated, cAMP-GEFII may

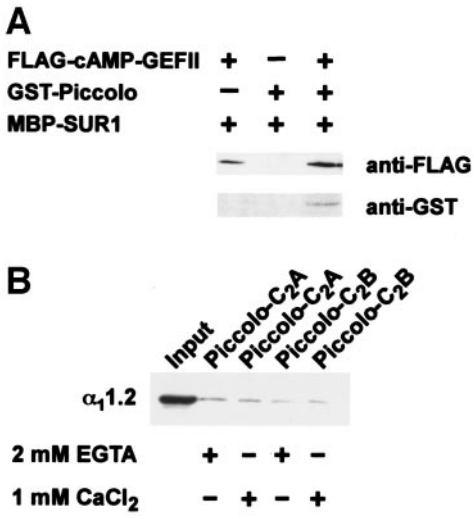

FIG. 2. Binding of SUR1, cAMP-GEFII, and Piccolo (*A*) and binding of Piccolo and VDCC (*B*). *A*: FLAG-cAMP-GEFII expressed in COS-1 cells was incubated with MBP-SUR1 (598–1,003) immobilized on amylose-resin, followed by incubation with GST-Piccolo (4,505–4,758). The samples were analyzed by immunoblotting with anti-GST antibody and anti-FLAG M2 antibody. In the left lane, MBP-SUR1 was incubated with FLAG-cAMP-GEFII. In the middle lane, MBP-SUR1 was incubated with GST-Piccolo without prior incubation of FLAG-cAMP-GEFII. In the right lane, MBP-SUR1 was incubated with FLAG-cAMP-GEFII, followed by incubation with GST-Piccolo. The data are representative of three experiments. *B*: GST-C_2 domains of Piccolo were incubated with Myc-tagged $\alpha_1$1.2-subunit (745–892) of L-type VDCC in the presence of 2 mmol/l EGTA or 1 mmol/l $CaCl_2$. The samples were analyzed by immunoblotting with anti-Myc antibody. The data are representative of two experiments.

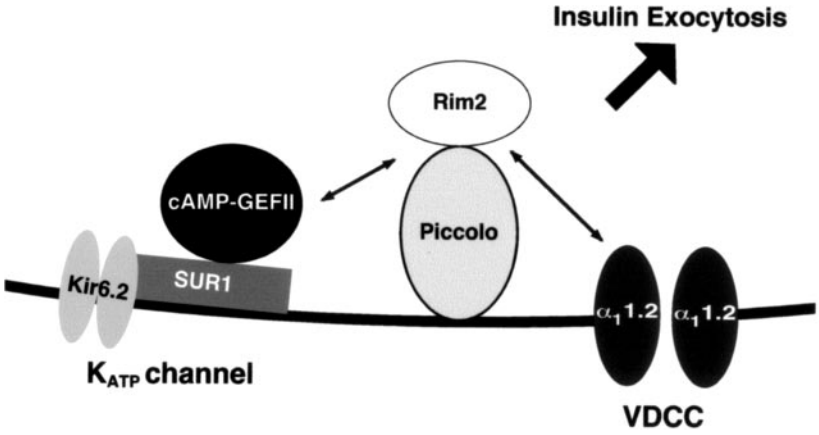

FIG. 3. Model of the integration of the ATP, cAMP, and Ca^{2+} signals in insulin granule exocytosis.

be anchored to SUR1 even when the ATP/ADP ratio is increased by the metabolism of glucose. This anchoring may be necessary to assemble the components in exocytosis, including the K_{ATP} channel, VDCC, Rim2, and Piccolo.

Incretins such as glucagon-like peptide (GLP)-1 and glucose-dependent insulinotropic polypeptide (GIP) increase the cAMP concentration in pancreatic β-cells. We previously found that incretin-induced, PKA-independent insulin secretion is mediated by cAMP-GEFII (17). Lack of interaction between SUR1 and cAMP-GEFII could disrupt assembly of the exocytotic components and lead to an impairment of cAMP-dependent, PKA-independent insulin secretion. This is supported by the finding that incretin-induced, PKA-independent insulin secretion is impaired in SUR1-deficient mice (24,25). We recently found that Piccolo in pancreatic β-cells interacts with cAMP-GEFII (18). Here we show that Piccolo interacts with SUR1 through cAMP-GEFII and with the $\alpha_1$1.2-subunit of L-type VDCC in a Ca^{2+}-independent manner.

Based on these findings, we propose a model of the interactions of ATP, cAMP, and Ca^{2+} sensors in the exocytosis of insulin granules, as shown in Fig. 3. NBF-1 of SUR1 interacts with cAMP-GEFII. cAMP-GEFII and SUR1 form a complex with Piccolo. Piccolo forms heterodimer with Rim2 in a Ca^{2+}-dependent manner. Both Piccolo and Rim2 interact with L-type VDCC. An increase in cAMP concentration upon stimulation may control the dissociation of cAMP-GEFII from SUR1. Clarification of the temporal and spatial interaction of these molecules in exocytosis of insulin granules requires further investigation.

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