Piccolo, a Ca$^{2+}$ Sensor in Pancreatic β-Cells

INVolvement of cAMP-GEFII-Rim2-PICCOLO Complex in cAMP-Dependent Exocytosis*

Received for publication, October 3, 2002
Published, JBC Papers in Press, October 24, 2002, DOI 10.1074/jbc.M210146200

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We have previously shown that cAMP-binding protein cAMP-guanidine nucleotide exchange factor II (GEFII) (or Epac2) interacting with Rim2 is involved in cAMP-dependent, protein kinase A-independent exocytosis in pancreatic β-cells. The action of the cAMP-GEFII-Rim2 complex requires both intracellular cAMP and Ca$^{2+}$. Although Rim2 has C$_2$ domains, its role as a Ca$^{2+}$ sensor has remained unclear. In the present investigation, we have discovered that Piccolo, a CAZ (cytoskeletal matrix associated with the active zone) protein in neurons that is structurally related to Rim2, also binds to cAMP-GEFII and that it forms both homodimer and heterodimer with Rim2 in a Ca$^{2+}$-dependent manner, whereas Rim2 alone does not form the homodimer. The association of Piccolo-Rim2 heterodimerization is stronger than Piccolo-Piccolo homodimerization. Treatment of pancreatic islets with antisense oligodeoxynucleotides against Piccolo inhibits insulin secretion induced by cAMP analog 8-bromo-cyclic AMP plus high glucose stimulation. These results suggest that Piccolo serves as a Ca$^{2+}$ sensor in exocytosis in pancreatic β-cells and that the formation of a cAMP-GEFII-Rim2-Piccolo complex is important in cAMP-induced insulin secretion. In addition, this study suggests that CAZ proteins similar to those in neurons are also function in pancreatic β-cells.

Stimulus secretion coupling is a crucial event in secretory cells including neurons and neuroendocrine and endocrine cells (1). In neurons, the plasma membrane of the presynaptic compartment contains the so-called active zone, a specialized region where synaptic vesicles dock and fuse (2, 3). The active zone is characterized ultrastructurally as an electron-dense region of cytoskeletal filaments under the plasma membrane where synaptic vesicles are clustered (4, 5). It is thought that the cytoskeletal matrix associated with the active zone (CAZ) plays a fundamental role in regulating the mobilization of synaptic vesicles and defining release sites (6). Recent studies of the active zone in neurons have found that CAZ proteins include Piccolo/aziconin (7, 8), Bassoon (9), Rim (10), Munc13-1 (11), and CAST (12).

Regulated exocytosis is a specialized feature of neurons and neuroendocrine and endocrine cells. These cells share various molecules that constitute the exocytotic machinery including SNARE proteins, synaptotagmins, and the members of the Rab3 subfamily (13). Although the active zone is well characterized in neurons, it is less understood in neuroendocrine and endocrine cells (14). The pancreatic β-cell is a typical endocrine cell in which exocytosis of the insulin-containing granules is regulated by a variety of factors. cAMP is a critical intracellular signal in the regulation of insulin secretion in pancreatic β-cells (15). The action of cAMP on insulin secretion generally has been thought to be mediated by phosphorylation of the many regulatory proteins by protein kinase A (16). We found recently that the cAMP-binding protein cAMP-GEFII (17), also referred to as Epac2 (18), is a direct target of cAMP in regulated exocytosis and that cAMP-GEFII interacting with Rim2 (19), a target of the small G-protein Rab3, mediates cAMP-dependent, protein kinase A-independent insulin secretion in pancreatic β-cells (20). The action of the cAMP-GEFII-Rim2 complex in insulin secretion requires increases in both intracellular cAMP and Ca$^{2+}$ (20). However, how cAMP and Ca$^{2+}$ signals are integrated in this mechanism is not clear. Because Rims (Rim1 and Rim2) have C$_2$ domains, it was initially postulated that the C$_2$ domains of Rims function as a Ca$^{2+}$ sensor in excitation-secretion coupling (10, 19, 20). However, recent studies indicate that Rim1 is not required for Ca$^{2+}$-regulated synaptic fusion (21, 22), suggesting that another molecule is a Ca$^{2+}$ sensor in the mechanism of cAMP-GEFII-mediated exocytosis in pancreatic β-cells.

In the search for a molecule in addition to Rim2 that couples directly to cAMP-GEFII, we found that the CAZ protein Piccolo (7), which is structurally related to Rim2, also binds to cAMP-

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The abbreviations used are: CAZ, cytoskeletal matrix associated with the active zone; SNARE, soluble N-sensitive factor attachment protein receptor; Epac, exchange protein directly activated by cAMP; ODN, oligodeoxynucleotide; 8-Br-cAMP, 8-bromo-cyclic AMP; GST, glutathione S-transferase; SPR, surface plasmon resonance; RIM-BP, Rim-binding protein; HA, hemagglutinin A; RT, reverse transcriptase; Pic, Piccolo; Piccolo-S, Piccolo-short form; Piccolo-L, Piccolo-long form; GEF, guanine nucleotide exchange factor; CAST, CAZ-associated structural protein.

‡ This work was supported in part by Grant-in-Aid 10NP0201 for Creative Basic Research and for Scientific Research from the Ministry of Education, Science, Sports, Culture, and Technology, a grant from the Foundation for Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation, and a grant from Novo Nordisk Pharma Ltd. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank accession number(s) AB083477 and AB083478.

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This paper is available on line at http://www.jbc.org
GEFII. We also found that Piccolo forms both the homodimer and heterodimer with Rim2 in a Ca\(^{2+}\)-dependent manner and that the association of the Piccolo-Rim2 heterodimerization is stronger than that of the Piccolo-Piccolo homodimerization in the presence of Ca\(^{2+}\). Treatment of pancreatic islets with antisense oligodeoxynucleotides (ODNs) against Piccolo inhibited insulin secretion induced by cAMP analog 8-bromo-cyclic AMP (8-BrcAMP) plus high glucose stimulation. We propose that Piccolo serves as a Ca\(^{2+}\) sensor in exocytosis in pancreatic β-cells and that the formation of the cAMP-GEFII-Rim2-Piccolo complex is important in the mechanism of cAMP-induced exocytosis.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screening and cDNA Cloning of Piccolo—**Yeast strain L40 (MATa trp1 leu2 his3 lys2::lexA HIS3 ura3::lexA lacZ) was transformed with a derivative of pBTM116 bearing a full-length cAMP-GEFII fused to the LexA DNA-binding domain (pBTM116-cAMP-GEFII) using the lithium acetate method (23). The screening of MIN6 cDNA library with the partial Piccolo (residues 4,505–2,466) or control ODNs was performed according to a surface, which was subtracted from each resonance measurement of GST fusion proteins.

**Northern Blotting and in Situ Hybridization—**Northern blot analysis was performed under standard stringent hybridization conditions with \(^32\)P-labeled 1,089-bp Piccolo cDNA. Membranes were washed with 0.1× SSC, 0.1% SDS at 50 °C for 60 min and exposed to x-ray film with intensifying screen at −80 °C. In situ hybridization was carried out as described previously (25) using the antisense oligonucleotide probe corresponding to bases 14,900–15,944.

**Coedemination and Immunoprecipitation Assays—**GST fusion proteins were purified according to the manufacturer’s instruction (Amer sham Biosciences). For coedemination assays, COS-1 cells were transfected with each plasmid using LipofectAMINE (Invitrogen). The lysate of COS-1 cells transfected with FLAG-tagged cAMP-GEFII and Piccolo or in buffer B (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM MgCl\(_2\), and 0.5% Nonidet P-40) for binding between cAMP-GEFII and Piccolo or in buffer B (50 mM HEPES, pH 7.4, 100 mM NaCl, and 1% Triton X-100) for binding between C2 domains and cleaved by centrifugation. This cellular lysate was incubated with GST fusion protein immobilized on glutathione-agarose for 90 min at 4 °C and washed extensively with each buffer four times. The eluates on the resins were eluted with SDS–PAGE sample buffer and subjected to immunoblotting.

**Identification of Piccolo and Its Interaction with cAMP-GEFII—**We performed a yeast two-hybrid screen with a full-length cAMP-GEFII as bait (19). The screening of MIN6 cDNA led to the isolation of partial Piccolo/aczonin (Piccolo) as well as Rim2. To obtain full-length PICOLO cDNA, we screened a MIN6 cDNA library with the partial Piccolo (residues 4,505–4,758) isolated by two-hybrid screen as a probe (Fig. 1A). Two transformants were isolated from the MIN6 cDNA library. We referred to as PICOLO-long form (PICOLO-L) and PICOLO-short form (PICOLO-S) (Fig. 1A). PICOLO-L has two C\(_2\) domains (C\(_2\)A and C\(_2\)B domains) in the carboxyl terminus, whereas PICOLO-S lacks the C\(_2\)B domain. Similar variants are expressed in mouse brain data have been submitted to GenBank™ data base under accession numbers AB0883477 and AB0883478, respectively.

**RESULTS**

**Identification of Piccolo and Its Interaction with cAMP-GEFII—**We performed a yeast two-hybrid screen with a full-length cAMP-GEFII as bait (19). The screening of MIN6 cDNA led to the isolation of partial Piccolo/aczonin (Piccolo) as well as Rim2. To obtain full-length PICOLO cDNA, we screened a MIN6 cDNA library with the partial Piccolo (residues 4,505–4,758) isolated by two-hybrid screen as a probe (Fig. 1A). Two transformants were isolated from the MIN6 cDNA library. We referred to as PICOLO-long form (PICOLO-L) and PICOLO-short form (PICOLO-S) (Fig. 1A). PICOLO-L has two C\(_2\) domains (C\(_2\)A and C\(_2\)B domains) in the carboxyl terminus, whereas PICOLO-S lacks the C\(_2\)B domain. Similar variants are expressed in mouse brain data have been submitted to GenBank™ data base under accession numbers AB0883477 and AB0883478, respectively. There is 93.7% amino acid identity between rat Piccolo-L and mouse Piccolo-L.

The lysate of COS-1 cells transfected with FLAG-tagged CAMP-GEFII was incubated with GST-tagged partial Piccolo (amino acid residues 4,505–4,758) (Fig. 1A). This partial Piccolo bound to FLAG-tagged cAMP-GEFII (data not shown). We then investigated to find whether the PDZ domain of Piccolo (Pic-PDZ) binds directly to cAMP-GEFII. GST-tagged Pic-PDZ bound to Myc-tagged CAMP-GEFII transfected in COS-1 cells (Fig. 1B). The binding of CAMP-GEFII to Piccolo was further confirmed using COS-1 cells cotransfected with Myc-tagged CAMP-GEFII and HA-tagged Pic-PDZ, indicating that CAMP-GEFII specifically binds to the PDZ domain of Piccolo.

**Tissue Expression of Piccolo—**Northern blot analysis revealed that Piccolo is expressed at high levels in cerebrum, cerebellum, and MIN6 cells and at moderate levels in pituitary gland, pancreatic islets, and pheochromocytoma-derived PC12 cells. In situ hybridization of mouse brain revealed that Piccolo mRNA is expressed in cerebellar cortex, hippocampus (especially CA3 and dentate gyrus), olfactory bulb, cerebellar cortex, and pituitary gland (Fig. 2B). The distribution of Piccolo mRNA largely overlaps with that of cAMP-GEFII and Rim mRNAs in tissues, cell lines, and mouse brain (19).
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Fig. 1. Binding of cAMP-GEFII to Piccolo through a PDZ domain both in vitro and in vivo. A, schematic representation of Piccolo-L, Piccolo-S, and mutants of Piccolo (Piccolo-(4,505–4,755)) and Pic-PDZ, Pic-C\(_{A}\), and Pic-C\(_{B}\) domains. B, In vitro binding between PDZ domain of Piccolo and cAMP-GEFII. GST alone or GST-tagged Pic-PDZ immobilized on glutathione-Sepharose was incubated with lysate of cells expressing Myc-tagged cAMP-GEFII. The samples were analyzed by immunoblotting (IB) with anti-Myc antibody. C, in vivo binding between PDZ domain of Piccolo and cAMP-GEFII. The cells coexpressing Pic-PDZ and cAMP-GEFII were harvested and lysed. The lysate was subjected to immunoprecipitation assay (IP) with anti-HA antibody and visualized by immunoblotting with anti-HA antibody.

Fig. 2. Expression of Piccolo. A, Northern blot analysis of Piccolo in various mouse tissues and mouse and rat endocrine- and neuroendocrine-derived cell lines. Lanes were loaded with 10 \(\mu\)g (5 \(\mu\)g for pancreatic islets) of total RNA from the indicated tissues and cell lines. Hybridizations and washing were carried out under standard conditions. Note that upper and lower panels were exposed for 72 h and 1 week, respectively. B, in situ hybridization of Piccolo in mouse brain. Upper panel shows cerebellum (Cb), caudoputamen (Cp), cortex (Ct), hippocampus (Hb), olfactory bulb (Ob), pons (Po), and thalamus (Th). Lower panel shows pituitary gland. Scale bars represent 1 mm.

Homodimerization and Heterodimerization of Piccolo and Rim2—C\(_{A}\) domains found in Ca\(^{2+}\)-binding proteins such as synaptotagmins are involved in Ca\(^{2+}\)-dependent homodimerization and heterodimerization (26). The dimerization of synaptotagmins through their C\(_{A}\) domains has been shown to be important for Ca\(^{2+}\)-induced exocytosis (27). We investigated to find whether the C\(_{A}\) domains of Piccolo dimerize in the presence of Ca\(^{2+}\). The constructs used are shown in Figs. 1A and 3A. The GST-tagged C\(_{A}\) or C\(_{B}\) domain of Piccolo (Pic-C\(_{A}\) or Pic-C\(_{B}\)) was incubated with lysate of COS-1 cells transfected with Myc-tagged C\(_{A}\) domain of Piccolo in the presence or absence of Ca\(^{2+}\). As shown in Fig. 3B, the C\(_{A}\) domain of Piccolo homodimerizes in the presence of Ca\(^{2+}\) but does not bind to the C\(_{B}\) domain of Piccolo. We also found that the C\(_{B}\) domain of Piccolo does not homodimerize in the presence of Ca\(^{2+}\) (data not shown). In addition, full-length Rim2 does not bind to either the C\(_{A}\) or C\(_{B}\) domain of Rim2 in the presence of Ca\(^{2+}\), indicating that Rim2 does not homodimerize.

We then determined whether the C\(_{A}\) domains of Rim2 bind to the C\(_{A}\) or C\(_{B}\) domain of Piccolo in a Ca\(^{2+}\)-dependent manner. GST-tagged Pic-C\(_{A}\) or Pic-C\(_{B}\) was incubated with the lysate of COS-1 cells transfected with full-length Rim2. As shown in Fig. 3C, the C\(_{A}\) domain of Piccolo binds to full-length Rim2 predominantly in the presence of Ca\(^{2+}\), whereas the C\(_{B}\) domain of Piccolo does not. To identify the region of Rim2 that binds to the C\(_{A}\) domain of Piccolo, GST-tagged Pic-C\(_{A}\) or Pic-C\(_{B}\) was incubated with the lysate of COS-1 cells transfected with Rim2 mutant lacking both C\(_{A}\) and C\(_{B}\) domains (Rim2\(_{3-3}\)). There was no interaction between Pic-C\(_{A}\) and Rim2\(_{3-3}\), indicating that the C\(_{A}\) domain of Piccolo binds to the C\(_{A}\) or the C\(_{B}\) domain of Rim2 or both (Fig. 3C). We then investigated Pic-C\(_{A}\) binding to Rim2-C\(_{A}\) or Rim2-C\(_{B}\). The GST-tagged C\(_{A}\) domain of Rim2 binds to Myc-tagged Pic-C\(_{A}\) in the presence of Ca\(^{2+}\), whereas the C\(_{B}\) domain of Rim2 does not (Fig. 4D). These results suggest that the C\(_{A}\) domain of Piccolo is involved in Ca\(^{2+}\)-dependent dimerization with the C\(_{A}\) domain of Piccolo or the C\(_{A}\) domain of Rim2.

To compare the effects of Ca\(^{2+}\) on the association between Pic-C\(_{A}\)-tagged Pic-C\(_{A}\)-homodimerization and Pic-C\(_{A}\)-tagged Rim2-C\(_{A}\)-heterodimerization, we employed SPR spectroscopy. Anti-GST antibodies were covalently coupled on a sensor chip, and then GST alone, GST-tagged Pic-C\(_{A}\), or GST-tagged Rim2-C\(_{A}\) was immobilized. The resonance units of GST alone or GST fusion proteins for anti-GST antibodies were nearly equal (data not shown), indicating that the anti-GST antibodies were equally saturated by GST and GST fusion proteins. The lysate of COS-1 cells transfected with Myc-tagged Pic-C\(_{A}\) was perfused across the sensor chip on which GST alone or GST-fused C\(_{A}\) domain was immobilized. The association of Pic-C\(_{A}\) with Pic-C\(_{A}\) or Rim2-C\(_{A}\) was confirmed by using SPR spectroscopy (Fig. 4A). The resonance units of the association between Pic-C\(_{A}\) and Rim2-C\(_{A}\) (heterodimerization) were twice as high as those of homodimerization of Pic-C\(_{A}\) (Fig. 4B), indicating that the association between the C\(_{A}\) domain of Piccolo and Rim2 (heterodimerization) is stronger than that of homodimerization of the C\(_{A}\) domain of Piccolo (Fig. 4B).

To determine whether the cAMP-GEFII-Rim2-Piccolo complex forms in the presence of Ca\(^{2+}\), FLAG-tagged cAMP-GEFII and Myc-tagged Rim2 were coexpressed in COS-1 cells and the lysate was incubated with GST-tagged Pic-C\(_{A}\) in the presence of Ca\(^{2+}\). As shown in Fig. 5, the C\(_{A}\) domain of Piccolo binds to the cAMP-GEFII-Rim2 complex in the presence of Ca\(^{2+}\) but not to cAMP-GEFII directly.

Piccolo Is Involved in cAMP-induced Exocytosis—Because cAMP-GEFII binds to the PDZ domain of Piccolo and mediates cAMP-induced exocytosis, we investigated to find whether Piccolo is involved in cAMP-GEFII in exocytosis. We examined the...
Effect of the PDZ domain of Piccolo on 8-Br-cAMP-induced exocytosis from MIN6 cells in which endogenous cAMP-GEFII and Piccolo are expressed. The PDZ domain of Piccolo was cotransfected with human preproinsulin in MIN6 cells. Because preproinsulin is converted into insulin and C-peptide during the secretory process in pancreatic β-cells and antibody against human insulin cross-react with endogenous mouse insulins (I and II), we monitored insulin secretion indirectly by measuring human C-peptide secretion from transfected cells. We previously showed that overexpression of Rim2ΔC2, which contains only a PDZ domain and lacks zinc finger and C2 domains, with human preproinsulin in MIN6 cells suppressed 8-Br-cAMP-induced C-peptide secretion (19, 20). The overexpression of the PDZ domain of Piccolo in MIN6 cells significantly suppressed 8-Br-cAMP-induced secretion of C-peptide in the presence of 16.7 mM glucose compared with controls transfected with luciferase (Fig. 6A). These results indicate that the interaction between cAMP-GEFII and Piccolo is involved in cAMP-induced exocytosis.

To clarify the physiological relevance of Piccolo, we investigated the role of endogenous Piccolo on insulin secretion. We utilized antisense ODNs against Piccolo in mouse pancreatic islets. We first ascertained whether treatment with antisense ODNs suppresses Piccolo at the protein level. Because anti-Piccolo antibody that can detect endogenous Piccolo is not available, we constructed partial Piccolo cDNA containing the recognition site for antisense ODN against Piccolo. COS-1 cells transfected with partial Piccolo were treated with antisense ODNs against Piccolo or control ODNs. Treatment with anti-

![Homodimerization or heterodimerization of Piccolo in vitro](image1)

Fig. 3. Homodimerization or heterodimerization of Piccolo in vitro. A, schematic representation of Rim2, mutants of Rim2 (Rim2ΔC2, Rim2-C2A, and Rim2-C2B), zinc finger, and PDZ, C2, and C2B domains. B, homodimerization of C2 domain of Piccolo and that of Rim2 in vitro. GST fusion proteins immobilized on glutathione-Sepharose were incubated with lysate of cells expressing Pic-C2A or Myc-Rim2. The samples were analyzed by immunoblotting (IB) with anti-Myc antibody. C, heterodimerization between Pic-C2A or Pic-C2B and Rim2 or Rim2ΔC2. D, heterodimerization between Rim2-C2A or Rim2-C2B and Pic-C2A.

![The effect of Ca2+ on the interaction between C2A domain of Piccolo and that of Piccolo or Rim2](image2)

Fig. 4. The effect of Ca2+ on the interaction between C2A domain of Piccolo and that of Piccolo or Rim2. Indicated GST fusion proteins were immobilized on a CM5 (Biacore) sensor chip covalently coated with anti-GST antibodies. Myc-tagged Pic-C2A was expressed in COS-1 cells, and lysate (Analyte) was prepared in running buffer in the presence of 1 mM CaCl2 or 2 mM EGTA and injected. A, representative data of sensogram. B, results are shown as the means ± S.E., n = 3 (*, p < 0.01).
sense ODNs markedly decreased the partial Piccolo protein level in transfected cells (Fig. 6B). We then evaluated the effect of antisense ODNs on 8-Br-cAMP-induced insulin secretion at high glucose (16.7 mM) from mouse pancreatic islets as described previously (20). 8-Br-cAMP-induced insulin secretion at high glucose was reduced significantly in the presence of antisense ODNs against Piccolo compared with treatment with control ODNs (Fig. 6C). These results indicate that Piccolo is involved in exocytosis induced by 8-Br-cAMP in pancreatic islets.

**mRNA Expressions of CAZ and CAZ-associated Proteins in Pancreatic Islets and MIN6 Cells—** In neurons, CAZ proteins (Piccolo, Bassoon, Rim1, Munc13-1, and CAST) and CAZ-associated proteins (prenylated rab acceptor-1, Profilin1, Profilin2, Rim-binding proteins (RIM-BP1 and RIM-BP2), and Liprin-α) are known to form multiple protein complexes at the active zone (12). All of these proteins with the exception of CAST and RIM-BP1 are expressed in pancreatic islets and MIN6 cells as assessed by RT-PCR analysis (Table I).

**DISCUSSION**

We recently showed that the cAMP-binding protein cAMP-GEFII interacting with Rim2 participates in cAMP-dependent, protein kinase A-independent exocytosis in pancreatic β-cells (19, 20). The action of the cAMP-GEFII-Rim2 complex in this process in pancreatic β-cells requires a rise in Ca\(^{2+}\) concentration as well as a rise in cAMP concentration (20). It is previously reported (28) also that cAMP induces insulin secretion in the presence of a high Ca\(^{2+}\) concentration but not in the presence of cAMP alone. These findings indicate that cAMP-induced insulin secretion in the mechanism of cAMP-dependent exocytosis in pancreatic β-cells is Ca\(^{2+}\)-dependent. Although Rim2 has C\(_2\) domains, it has remained unclear how Rim2 functions as a Ca\(^{2+}\) sensor. During the search for molecules interacting with cAMP-GEFII in MIN6 cells, we found that Piccolo binds directly to cAMP-GEFII. Similar to Rim1 (10), Piccolo in neurons is a CAZ protein (7). Interestingly, Piccolo and Rim1 are structurally similar in that they have zinc finger domain(s), a PDZ domain, and C\(_2\) domains (7, 10). C\(_2\) domains found in Ca\(^{2+}\)-binding proteins are thought to be involved in Ca\(^{2+}\)-dependent homodimerization or heterodimerization (26). For example, the dimerization of synaptotagmins through their C\(_2\) domain is important in their Ca\(^{2+}\)-sensing function in Ca\(^{2+}\)-triggered exocytosis (28). Therefore, we investigated the homodimerization and heterodimerization of Piccolo and Rim2. We found that Rim2 cannot form homodimer, whereas Piccolo can form both homodimer and heterodimer with Rim2 in a Ca\(^{2+}\)-dependent manner. These results are consistent with previous reports that putative Ca\(^{2+}\) binding sites in the C\(_2\)A domain of Piccolo are similar to those in the C\(_2\)A domain of synaptotagmin 1 and that the C\(_2\) domains of Rim1 lack canonical aspartate residues involved in putative Ca\(^{2+}\) binding sites (29, 30). The association of the Piccolo-Rim2 complex is stronger than that of the Piccolo-Piccolo complex in the presence of Ca\(^{2+}\) as assessed by SPR spectroscopy. Considering these findings together, it is probable that Piccolo rather than Rim2 functions as a Ca\(^{2+}\) sensor in the mechanism of cAMP-induced exocytosis in pancreatic β-cells. The properties of the complex of cAMP-GEFII, Rim2, and Piccolo such as the stoichiometry are unclear at present. It has been suggested that Piccolo is an unusual Ca\(^{2+}\) sensor with Ca\(^{2+}\) affinity (\(K_d \sim 1.5 \text{ mM}\)) too low to trigger release in neurons but ideally suited for a such a role in Ca\(^{2+}\) accumulation through repetitive stimulation (30). It is known that the rise in [Ca\(^{2+}\)]\(i\), elicited during stimulation by cAMP plus high glucose in pancreatic β-cells is not transient but is sustained (31), suggesting the accumulation of [Ca\(^{2+}\)]\(i\) during the stimulation. Indeed, the treatment of pancreatic islets...
islets with antisense ODNs against Piccolo significantly inhibited insulin secretion induced by 8-Br-cAMP plus high glucose. This is compatible with the function of Piccolo as a Ca\(^{2+}\) sensor in cAMP-induced exocytosis of insulin granules. Several isoforms of synaptotagmins, which are thought to serve as a Ca\(^{2+}\) sensor for fast Ca\(^{2+}\)-triggered release in neurons, also are expressed in pancreatic \(\beta\)-cells (32). It is tempting to speculate that Piccolo and synaptotagmins function as distinct Ca\(^{2+}\) sensors in Ca\(^{2+}\)-triggered exocytosis of insulin granules in pancreatic \(\beta\)-cells, responding to different stimuli. It also is possible that the heterodimerization of Piccolo and Rim2 might alter the Ca\(^{2+}\) affinity of Piccolo according to SPR spectroscopy results. Recently, a model for the calcium microdomain has been proposed in neurons (33). The Ca\(^{2+}\) concentration has been calculated to reach millimolar order in a subcellular region proximal to the Ca\(^{2+}\) channel site (33). It has been suggested that 1-type Ca\(^{2+}\) channels colocalize with insulin-containing secretory granules in mouse pancreatic \(\beta\)-cells (34). The K/K/R/K/R/T sequence in the C\(_2\) domains of Rim1 and synaptotagmin 1 that bind to the \(\alpha_1\)-subunits of the Ca\(^{2+}\) channels (35) also is conserved in the C\(_2\)B domain of Piccolo. This suggests that Piccolo is associated with the putative calcium microdomain in pancreatic \(\beta\)-cells. In neurons, CAZ proteins (Piccolo, Bassoon, Rim1, Munc13-1, and CAST) have been characterized and implicated in forming multiple protein complexes at the active zone (12). All of the CAZ proteins and CAZ-associated proteins in neurons are expressed in pancreatic islets and MIN6 cells with the exception of CAST in pancreatic islets (32). It is tempting to speculate that the active zone also is a feature of pancreatic \(\beta\)-cells. Further biochemical and morphological studies are necessary to establish the fundamental structure and molecular basis of the exocytotic machinery of pancreatic \(\beta\)-cells as typical large dense core vesicle-containing endocrine cells.

Acknowledgments—We thank Nobuaki Ozaki for the involvement of initial cloning of Piccolo cDNA. We also thank Takaaki Ohashi for helpful advice about SPR analysis. We are grateful to Osamu Ohara for providing us with human Piccolo cDNA (KIAA0559).

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Piccolo, a Ca\(^{2+}\) Sensor in Pancreatic \(\beta\)-Cells: INVOLVEMENT OF cAMP-GEFII-Rim2·PICCOLO COMPLEX IN cAMP-DEPENDENT EXOCYTOSIS

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*J. Biol. Chem. 2002, 277:50497-50502.*

doi: 10.1074/jbc.M210146200 originally published online October 24, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M210146200

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