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The CD38-cyclic ADP-ribose signalling system in insulin secretion: molecular basis and clinical implications

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In answer to the comments of Islam and Berggren concerning our hypothesis on the CD38-cyclic ADP-ribose (cADPR) signalling system, we will present several lines of evidence that we believe can explain the discrepancies between their view and ours.

The Okamoto model and cADPR

Glucose is the primary stimulus of insulin secretion and synthesis in pancreatic beta cells of the islets of Langerhans [1–3]. Increases in the intracellular Ca^{2+} concentration mediate the biochemical events that couple glucose stimulation to insulin secretion, and mobilization of Ca^{2+} from intracellular stores in the endoplasmic reticulum as well as Ca^{2+} influx from extracellular sources are important in this process [4]. Concerning the mechanism of Ca^{2+} influx from extracellular sources, it has been proposed that ATP generated in the process of glucose metabolism inhibits the ATP-sensitive K^+ channel, causing beta cell membrane depolarization, thereby opening the voltage-dependent Ca^{2+} channel and resulting in Ca^{2+}-influx from the extracellular space [5]. However, it was thought that inositol 1, 4, 5-trisphosphate (IP_3) is a second messenger for Ca^{2+} mobilization from intracellular stores [6]. Since 1981, we have proposed a model for beta cell damage and its prevention as shown in Figure 1; that is, diabetogenic agents such as streptozotocin and alloxan damage DNA, activating poly(ADP-ribose) synthetase which uses NAD^+ as a substrate [2, 7–12]. Consequently, intracellular levels of NAD^+ fall dramatically, causing the inhibition of cellular functions such as insulin synthesis and secretion, and the beta cell ultimately dies. Although insulin-dependent diabetes mellitus (IDDM) can be caused by many different agents such as immunologic abnormalities, inflammatory tissue damage and beta-cytotoxic chemical substances, the final pathway for the toxic agents is the same, as shown in Figure 1. Therefore, IDDM is theoretically preventable by suppressing immune reactions, scavenging free radicals, and inhibiting the poly (ADP-ribose) synthetase by nicotinamide and 3-aminobenzamide. The question then arises as to why maintaining the cellular NAD^+ level is essential for beta cells to secrete and synthesize insulin. Since 1987, a metabolite of NAD^+, cADPR, has been reported to be as potent and powerful a releaser of intracellular Ca^{2+} as IP_3 in a variety of cells [13–29]. In 1993, we found that the cADPR level increased in pancreatic islets in response to glucose stimulation and that cADPR released Ca^{2+} from the microsomes, suggesting that cADPR has a second messenger role in insulin secretion [15]. Furthermore, we found that the cADPR level was not increased by glucose stimulation in pancreatic islets pretreated with streptozotocin [15]. When poly(ADP-ribose) synthetase inhibitors such as nicotinamide and 3-aminobenzamide were present, the cADPR level increased with glucose stimulation even in the presence of streptozotocin. These results suggest that cADPR is synthesized from NAD^+ by glucose stimulation in beta cells [12, 15]. We have thus proposed another pathway, the CD38 (ADP-ribosyl cyclase/cADPR

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Abbreviations. cADPR, cyclic ADP-ribose; IP_3, inositol 1, 4, 5-trisphosphate; RyR, ryanodine receptor Ca^{2+} channel; RT-PCR, reverse transcription polymerase chain reaction; RIA, radioimmunoassay; FSBA, 5′-p-fluorosulfonylbenzoyladenosine; KIC, α-ketoisocaproate; BST-1, bone marrow stromal antigen 1; PCR, polymerase chain reaction; SSCP, single stranded conformation polymorphism; FKBP12, FK506-binding protein 12; FKBP12.6, FK506-binding protein 12.6; CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; CICR, Ca^{2+}-induced Ca^{2+} release; IDDM, insulin-dependent diabetes mellitus.

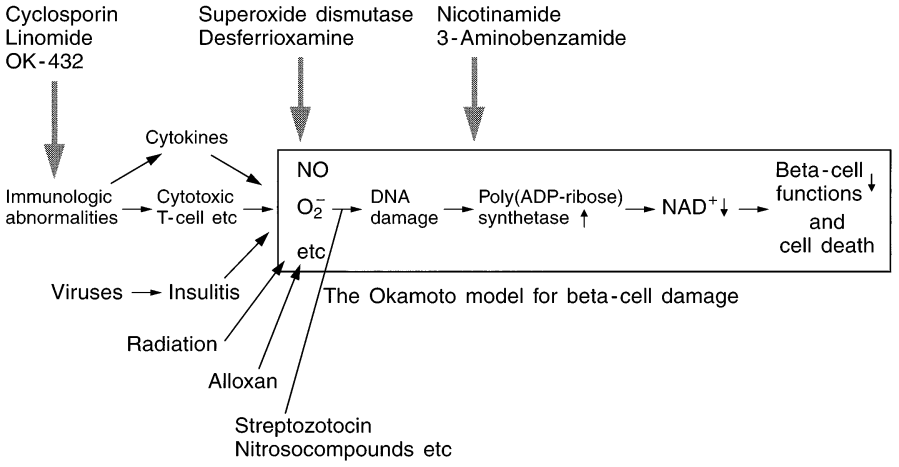

Fig. 1. A unifying model for beta-cell damage and its prevention in toxin- or virus-induced and immune diabetes (The Okamoto model) (adapted from [11, 30]). Although IDDM can be caused by many different agents such as immunologic abnormalities, inflammatory tissue damage, alloxan and streptozotocin, the final pathway leading to beta-cell damage is the same. This pathway involves the generation of free radicals, DNA damage, nuclear poly (ADP-ribose) synthetase activation and NAD^+ depletion. The fall in cellular NAD^+ inhibits the cellular functions such as insulin synthesis and secretion, and the beta-cell ultimately dies. Therefore, the beta-cell damage is theoretically preventable through inhibition of the serial reactions, as indicated by shaded arrows. One method is by inhibiting abnormal immune reactions with immunomodulators such as cyclosporin, linomide and OK-432 [68, 69]. Others are scavenging the radicals, which break DNA, by superoxide dismutase and other radical scavengers and inhibiting the poly (ADP-ribose) synthetase by specific inhibitors such as nicotinamide and 3-aminobenzamide to prevent the decrease in the NAD^+ level

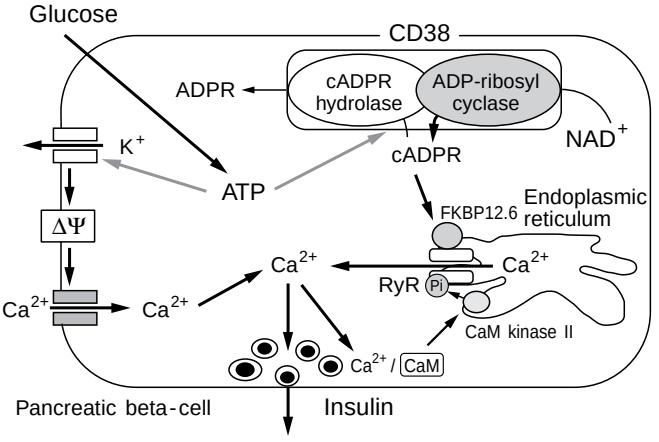

Fig. 2. Insulin secretion by glucose stimulation in beta cells (adapted from [36]). The insulin secretion via the CD38-cADPR signalling system as described in this paper is shown on the right. cADPR binds to FKBP12.6 to release Ca^{2+}, dissociating FKBP12.6 from RyR [35]. CaM kinase II phosphorylates RyR to sensitize and activate the Ca^{2+} channel (*Pi,* phosphorylation of RyR by CaM kinase II) [22]. Ca^{2+}, released from intracellular stores and/or supplied from extracellular sources, further activates CaM kinase II and amplifies the process. In this way, Ca^{2+}-induced Ca^{2+} release (CICR) can be explained. The conventional insulin secretion mechanism by Ca^{2+} influx from extracellular sources [5, 70] is shown on the left. ADPR, ADP-ribose

hydrolase) – cADPR signal system, for the increase in the intracellular Ca^{2+} concentration for insulin secretion by glucose in pancreatic beta cells [15, 30, 31]: millimolar concentrations of ATP, generated in the process of glucose metabolism, induce cADPR accumulation in beta cells by inhibiting the cADPR hydrolase activity of CD38 [32–34], and cADPR then acts as a second messenger for intracellular Ca^{2+} mobilization from the endoplasmic reticulum for insulin secretion [15, 22, 35, 36] (Fig. 2).

Ca^{2+} release by cADPR from islet microsomes

We have established a method for isolating large numbers of islets from pancreases, and the hand-picked islets after the collagenase digestion were shown to retain fully the functions of insulin secretion and synthesis in response to glucose stimulation [2]. We examined the release of Ca^{2+} from rat pancreatic islet microsomes and found that cADPR induced Ca^{2+} release from islet microsomes but IP_3 did not [15]. In rat cerebellum microsomes, both cADPR and IP_3 induced Ca^{2+} release, and heparin, an inhibitor of IP_3 binding to its receptor, blocked only the IP_3-induced Ca^{2+} release [15]. These results indicate that cADPR is effective in Ca^{2+} release from islet microsomes. In contrast, in cerebellum microsomes, both cADPR and IP_3 induce Ca^{2+}, but the mechanisms of Ca^{2+} release and/or the Ca^{2+} pools appeared to be different from each other.

Some controversial results have been reported using diabetic beta cells such as *ob/ob* mouse islets and RINm5F cells [37–39]. We have recently examined the Ca^{2+} releasing activity of these diabetic beta cell microsomes, compared it to that of the

microsomes of normal beta cells such as C57BL/6J mouse islets, and found that the Ca^{2+} release responses of these diabetic beta cell microsomes were quite different from those of normal islet microsomes [36]. Microsomes from C57BL/6J mouse islets as well as Wistar rat islets released Ca^{2+} in response to cADPR but scarcely in response to IP_3. This response to cADPR was completely attenuated by the prior addition of 100 nmol/l 8-amino (NH_2)-cADPR, an antagonist of cADPR [29, 40]. In contrast to normal islet microsomes, *ob/ob* mouse islet microsomes released only a small amount of Ca^{2+} by cADPR but released more Ca^{2+} by IP_3. RINm5F cell microsomes responded well to IP_3 to release Ca^{2+} but did not respond to cADPR. The Ca^{2+} releases by IP_3 in *ob/ob* mouse islets and RINm5F microsomes were attenuated by the prior addition of 100 μg/ml heparin and the small Ca^{2+} release response to cADPR observed in *ob/ob* islet microsomes was also attenuated by the prior addition of 8-NH_2-cADPR. These results together with the finding that *ob/ob* mouse beta cells and RINm5F cells released Ca^{2+} in response to IP_3 but scarcely in response to cADPR by patch clamp experiments [37–39] strongly suggest that cADPR acts as a second messenger for Ca^{2+} mobilization from intracellular stores in normal beta cells and that the Ca^{2+} release machinery by cADPR may be replaced with that by IP_3 in diabetic beta cells.

Moreover, concerning intracellular Ca^{2+} release channels, the mRNA expression of the type 2 ryanodine receptor (RyR-2), which is postulated to be a Ca^{2+} release channel for cADPR [35, 41], was clearly detected in normal mouse (C57BL/6J) islets but not in *ob/ob* mouse islets by reverse transcription-polymerase chain reaction (RT-PCR). In contrast, IP_3 receptor (IP_3R-1, IP_3R-2, IP_3R-4 and IP_3R-5) mRNAs were not detected in normal islets but were clearly detected in *ob/ob* islets, and although IP_3R-3 mRNA was slightly detected in normal islets, the mRNA expression was significantly increased in *ob/ob* islets. The expression of IP_3R-3 in normal rat islets and its increased expression in RINm5F cells have also been reported [42]. These changes in the gene expression of intracellular Ca^{2+} release channels fit well with the observation that IP_3-induced Ca^{2+} mobilization preferentially works in *ob/ob* islet and RINm5F cell microsomes. From these results, it is possible that changes in the gene expression involved in intracellular Ca^{2+} mobilization occur in diabetic beta cells, resulting in an abnormal response to glucose.

cADPR accumulation in islets in response to glucose stimulation

The next important issue is whether the accumulation of cADPR is actually caused by glucose stimulation in pancreatic islets. Recently, we incubated normal rat (Wistar) islets with 2.8 and 20 mmol/l glucose, and assayed the cADPR content in the islets by radioimmunoassay (RIA) using an anti-cADPR antibody [36, 43]. The cADPR content of islets incubated with 20 mmol/l glucose was increased within 5 min, whereas the cADPR content of islets incubated with 2.8 mmol/l glucose was not. The results were consistent with our previous observation based on the Ca^{2+} releasing activity of islet extracts [15]. Malaisse et al. [44] measured the cADPR content in rat islets and reported that it appeared not to be significantly affected by glucose. In our experiments, fasting of rats before isolation of the islets and the usage of Hanks solution containing 2.8 mmol/l glucose during the islet isolation may account for the rapid and significant increase of the cADPR content in islets in response to glucose stimulation. Furthermore, we determined the cADPR content by assessing the recovery of cADPR in the extraction and concentration procedures, but they did not. On the other hand, Malaisse et al. [44] incubated islets for a relatively longer period (90 min), whereas we incubated them for 5–40 min. In our experiment, the cADPR content in islets increased significantly within 10 min of incubation with high glucose but the small increase after 40 min incubation was not statistically significant compared with the cADPR content in islets incubated with low glucose. These differences in the experimental conditions may be responsible for the different results. We also isolated islets from C57BL/6J mice, incubated them under low and high glucose conditions, and measured the cADPR content. The cADPR content was significantly increased by glucose stimulation. In *ob/ob* mouse islets, a small amount of cADPR was detected when incubated under low glucose conditions but the cADPR content was not increased by high glucose. Furthermore, we examined the cADPR content of RINm5F cells. cADPR was not detectable in RINm5F cells even by glucose stimulation. We have already examined the effects of cADPR and IP_3 on insulin secretion by using digitonin-permeabilized pancreatic islets of normal Wistar rats [15]. Both cADPR and Ca^{2+} induced insulin secretion, but IP_3 did not. The combined addition of cADPR and Ca^{2+} did not induce significantly more insulin secretion than the addition of cADPR or Ca^{2+} alone. The cADPR-induced insulin secretion was inhibited by the addition of EGTA. The results so far described suggest that cADPR acts as a second messenger for Ca^{2+} release for insulin secretion in normal beta cells by glucose stimulation, but not in diabetic beta cells.

cADPR metabolizing enzymes

We and others have found that human [32, 45], mouse [46] and rat [47] CD38s have both ADP-ribosyl cyclase, synthesizing cADPR from NAD^+, and cADPR

hydrolase to produce ADP-ribose. We expressed human CD38 cDNA in *Escherichia coli,* purified the CD38 protein and confirmed that CD38 has both ADP-ribosyl cyclase and cADPR hydrolase activities [34]. We further found that millimolar concentrations of ATP markedly inhibited the cADPR hydrolase of CD38. Incubation of CD38 with NAD^+ in the presence of ATP led to an accumulation of cADPR in the incubation medium [32, 34]. Examination of the ATP binding site using an ATP analogue, 5′-p-fluorosulfonylbenzoyladenosine (FSBA) revealed that FSBA bound to lysine 129 of CD38. The cADPR hydrolase activity of CD38 was shown to be inhibited by ATP in a competitive manner with cADPR [34]. Therefore, ATP, produced in the process of glucose metabolism, appears to compete with cADPR for lysine 129 to inhibit the cADPR hydrolase activity of CD38, thereby increasing the cADPR content in islets as described above (see also Fig. 2).

We prepared transgenic mice overexpressing CD38 in pancreatic beta cells by ligating the rat insulin promoter to human CD38 cDNA and microinjecting it into fertilized mouse eggs [33]. The transgenic mice secreted more insulin than normal mice in response to glucose concentrations. The cADPR hydrolase activity of CD38 in the transgenic islets was inhibited by millimolar concentrations of ATP. It should be noted here that, in the transgenic islets, α-ketoisocaproate (KIC)-induced insulin secretion was also elevated compared with that in normal mouse islets. KIC, a deaminated metabolite of leucine, moves into the TCA cycle and rapidly produces ATP. However, tolbutamide and potassium chloride, which induce insulin secretion by promoting Ca^{2+} influx from extracellular sources, showed no significant difference in insulin secretion between transgenic and normal islets [33]. These results also provided further evidence for the contribution of the CD38-cADPR signal system to the release of Ca^{2+} from intracellular Ca^{2+} stores.

Kaisho et al. [48] found that the amino acid sequence of bone marrow stromal cell antigen 1 (BST-1) had significant homology (33 % identity) with that of CD38. In an experiment using recombinant BST-1 protein, BST-1 showed only low enzymic activities of ADP-ribosyl cyclase and cADPR hydrolase in the presence of Zn^{2+} and Mn^{2+} or under acidic conditions [49], suggesting that BST-1 may play a role as a surface antigen rather than as an enzyme [50].

CD38 mRNA was detected in almost all human tissues, and especially high levels of the mRNA expression were observed in high glucose-utilizing tissues such as liver, kidney and brain [32]. We also cloned rat CD38 cDNA and determined the primary structure of the protein. Rat CD38 mRNA was expressed in pancreatic islets [47]. However, in RINm5F cells, rat insulinoma-derived immortal cells, which show almost no glucose-induced insulin secreting ability, CD38 mRNA was not expressed [29, 47, 50]. Furthermore, the CD38 mRNA level was significantly decreased in *ob/ob* islets. The decreased CD38 mRNA in *ob/ob* islets may explain the low response in cADPR content by glucose stimulation. Decreased CD38 mRNA was also reported in islets of Goto-Kakizaki (GK) diabetic rats [51], which show impaired glucose-induced insulin secretion [52].

CD38 was originally identified as a human leukocyte surface antigen and suggested to catalyse the enzymic activity at the extracellular domain. CD38 was however expressed not only in the plasma membrane but also in microsomal membrane fractions [32, 33]. In addition, a cADPR metabolizing enzyme purified from canine spleen microsomes was identified as the canine homologue of CD38 [53], and an increase of the cADPR content in HL-60 cells corresponding to the induction of CD38 by retinoic acid was reported [43]. Our recent experiments indicated that the cADPR levels in CD38 cDNA-introduced RINm5F clones 1 and 3, RINm5F-derived cell lines into which CD38 had been introduced, were significantly higher than those of RINm5F cells, in which CD38 mRNA expression was not detected [47, 50].

We determined the nucleotide sequences of *Aplysia kurodai* ADP-ribosyl cyclase [54] and human CD38 genes [55]. Both genes contain 8 exons and 7 introns, and the exon-intron boundaries are well-conserved between both genes. Ten cysteine residues, which are conserved in the two genes and are thought to be essential for the enzyme activity, were coded to each corresponding exon [55]. It could be speculated that both genes have evolved from a common ancestral gene. We mapped the human CD38 gene to chromosome 4p15 [56]. We have experimentally demonstrated that one amino acid mutation of CD38 causes a great change in the cADPR metabolizing enzyme activity of CD38 [34, 57]. Thus, we searched for CD38 gene mutations in diabetic patients. In 100 non-insulin-dependent diabetic patients with insufficient insulin secretion and 90 control subjects, we amplified all 8 exons of the CD38 gene by PCR, screened the gene mutations by the single strand conformation polymorphism (SSCP) method and determined the sequences of the mutated gene. No mutation causing any amino acid change in CD38 was observed in the control group, but a gene mutation that caused the replacement of arginine 140 of CD38 by tryptophan was observed in four patients in the diabetes group. We actually expressed the mutated gene in COS-7 cells, measured the cADPR metabolizing enzyme activity and found that the enzyme activity (ADP-ribosyl cyclase/cADPR hydrolase) of the mutated protein was reduced to 40–50 % of the normal CD38 activity [58].

Mechanism of Ca^{2+} release by cADPR

As described above, cADPR is considered to mediate the release of Ca^{2+} from the endoplasmic reticulum by a mechanism different from that of IP_3. The addition of ryanodine to islet microsomes caused Ca^{2+} release, while the addition of cADPR to ryanodine-treated microsomes did not [15]. These results suggested that the cADPR-mediated Ca^{2+} release was caused via the RyR in the endoplasmic reticulum. We expressed 3 types (type 1, 2, and 3) of rabbit RyRs in COS-7 cells to perform a binding experiment with cADPR, but it did not bind to any expressed channel protein. Therefore, cADPR appeared to act on RyR through some mediators to release Ca^{2+}. RyRs have been purified from both skeletal and cardiac muscle [59, 60], and FK506 (an immunosuppressant)-binding protein 12 (FKBP12) and FK506-binding protein 12.6 (FKBP12.6) were co-purified with type 1 RyR from striated muscle and with type 2 RyR from cardiac muscle, respectively [61, 62]. FKBP12 and FKBP12.6 were shown to bind selectively to type 1 and type 2 RyR, respectively [63]. It was further reported that the type 1 RyR was activated by dissociation of FKBP12 from the RyR by the addition of FK506 to release Ca^{2+} [64]. Recently, we found that FK506 as well as cADPR induced the release of Ca^{2+} from islet microsomes [35]. After islet microsomes were treated with FK506, the Ca^{2+} release by cADPR from the microsomes was reduced. cADPR as well as FK506 bound to FKBP12.6, which we also found occurs naturally in islet microsomes. When islet microsomes were treated with cADPR, FKBP12.6 dissociated from the microsomes and moved to the supernatant, releasing Ca^{2+} from the intracellular stores. The microsomes that were then devoid of FKBP12.6 did not show Ca^{2+} release by cADPR. Our recent experiments indicated that type 2 RyR is expressed in rat and mouse islets [35, 36]. From these results, it is strongly suggested that cADPR may be a ligand for FKBP12.6 in islet RyR and that, when cADPR binds to FKBP12.6 in islet microsome RyR and causes the dissociation of FKBP12.6 from the RyR to form FKBP12.6-cADPR complex, the channel activity of the RyR is thereby increased to release Ca^{2+} from the endoplasmic reticulum [35]. The fact that rat FKBP12.6 mRNA was not expressed in RINm5F cells [35] may also explain the negative Ca^{2+} release response to cADPR of RINm5F cells [37–39].

In sea urchin eggs, it was suggested that calmodulin directly interacts with RyR to enhance the cADPR-mediated Ca^{2+} release [20, 21]. We found that calmodulin greatly sensitized and enhanced the cADPR-mediated Ca^{2+} release from islet microsomes [22]. Furthermore, inhibitors for calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) completely abolished the glucose-induced insulin secretion as well as the cADPR-mediated and calmodulin-enhanced Ca^{2+} release. These results suggested that the enhancing effect of calmodulin is due to phosphorylation of RyR by CaM kinase II in islet microsomes [22]. As calmodulin can easily be diluted under whole cell patch conditions, the negative Ca^{2+} release responses to cADPR obtained by the whole cell patch technique [37–39] may be explained by the loss or lack of calmodulin in the patch. As described above, the RyR can also be activated by the dissociation of FKBP12.6. The interaction between the dissociation of FKBP12.6 from RyR and the phosphorylation of RyR by CaM kinase II remains to be elucidated.

cADPR in other cells and future perspectives

In this paper we have described a novel mechanism of insulin secretion, the CD38-cADPR signalling system (Fig. 2). An increase in intracellular $[Ca^{2+}]$, which then triggers insulin secretion, has conventionally been explained by the hypothesis put forth by Ashcroft et al. [5] of Oxford University, in which Ca^{2+} influx is provided by extracellular sources [5]. The present paper describes a novel mechanism of insulin secretion in which the Ca^{2+} release from the endoplasmic reticulum, an intracellular Ca^{2+} pool, induces insulin secretion. Rojas et al. [4] examined the Ca^{2+} influx from extracellular sources and the Ca^{2+} release from the intracellular pool in human beta cells, and showed that 42–75 % of the increase in intracellular $[Ca^{2+}]$ by glucose stimulation was due to the release of Ca^{2+} from the intracellular stores. Thus, the importance of Ca^{2+} release from the endoplasmic reticulum in insulin secretion by glucose has been recognized. In the CD38-cADPR signalling system, an abnormality in Ca^{2+} release from RyR may cause a decrease in insulin secretion, leading to the development and exacerbation of diabetes. As oral therapeutic agents for diabetes, sulfonylureas such as tolbutamide, which promotes Ca^{2+} influx from extracellular sources, have been used. As described above, the Ca^{2+} release from intracellular stores is also regarded as important in glucose-stimulated insulin secretion. Thus, agents promoting Ca^{2+} release from the endoplasmic reticulum may prove to be effective in treating diabetes.

IP_3 has been considered to be a second messenger for intracellular Ca^{2+} mobilization [6]. As described in this paper, in normal pancreatic beta cells, cADPR acts on the endoplasmic reticulum to release Ca^{2+}. In this case, it appears that cADPR binds to a protein, FKBP12.6, and dissociates the protein from RyR to release Ca^{2+}. Recently, various physiological phenomena from animal to plant cells, in addition to those of pancreatic beta cells, become understandable in terms of the cADPR-mediated Ca^{2+} mobilization [15–29, 65]. The list could be longer. Thus, cells can utilize two major second messengers, IP_3 and

cADPR, for Ca^{2+} mobilization depending on the species of cells and differences in the cellular conditions, physiological or pathological, and perform a variety of cellular functions.

Ca^{2+}-induced Ca^{2+} release (CICR) is an important physiological phenomenon observed first in skeletal muscle cells [66, 67]. Subsequently, CICR has become known as an important regulatory mechanism of intracellular $[Ca^{2+}]$ in almost all cells. As described in this paper, CICR can be explained by a series of amplification processes in which CaM kinase II is activated by Ca^{2+} released from intracellular stores and/or supplied from extracellular sources, and the activated enzyme phosphorylates RyR to further release Ca^{2+} from the activated RyR (see also Fig. 2). The fact that phosphorylation can be involved in the process of the CD38-cADPR signalling system suggests that this novel signalling system can be involved in long-term cell phenomena such as cell proliferation and plasticity of nerve cells, as well as relatively short-term physiological phenomena such as insulin secretion.

Note added in proof: Reference 36 is a review paper, experimental details of which will be published (Takasawa S, Akiyama T, Nata K et al. (1998) Cyclic ADP-ribose and inositol 1, 4, 5-trisphosphate as alternate second messengers for intracellular Ca^{2+} mobilization in normal and diabetic β-cells. J Biol Chem in press).

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