Cyclic ADP–Ribose in β Cells

Shin Takasawa *et al.* (*1*) challenge the role of inositol 1,4,5-triphosphate (IP_3) as an intracellular second messenger that mobilizes Ca^{2+} in pancreatic β cells. They found that cyclic adenosine diphosphate–ribose (cADP-ribose), but not IP_3, releases Ca^{2+} from islet microsomes. It is difficult to reconcile their results with many studies that establish IP_3 as an intracellular Ca^{2+}-mobilizing second messenger in pancreatic β cells (*2*).

Confronted with such provocative results, we performed a series of experiments to compare the Ca^{2+}-mobilizing actions of the two second messengers in β cells. We used clonal insulin-secreting RINm5F cells and cells obtained from *ob/ob* mice, where more than 95% of the islet cells correspond to normal β cells. The cells were permeabilized by high-voltage electric discharges, a technique that creates clean holes in the plasma membrane, but leaves intracellular Ca^{2+}-storing organelles in situ and undamaged (*3*). We found pronounced Ca^{2+} release when IP_3 was added to insulin-secreting RINm5F cells (Fig. 1A) or to pancreatic β cells from *ob/ob* mice (Fig. 1B). In marked contrast to the results in the report by Takasawa *et al.*, there was no Ca^{2+} release after the addition of cADP-ribose.

In experiments with β cells, we first added a low dose of caffeine to sensitize the release mechanism that presumably might respond to cADP-ribose. After maximal Ca^{2+} release by IP_3, further Ca^{2+} was released from β cells by the sulfhydryl reagent thimerosal which, as we have shown before, indicates the possible existence of a Ca^{2+}-induced Ca^{2+} release mechanism in β cells (*4*). With the use of intact β cells, we looked for the caffeine-sensitive intracellular Ca^{2+} pool on which cADP-ribose presumably acts. In small clusters of β cells that had been loaded with Fura-2, in the absence of extracellular Ca^{2+}, there was marked Ca^{2+} release from intracellular stores by IP_3-forming agonists, whereas caffeine-induced Ca^{2+} release was absent (*5*). Detailed studies using caffeine indicate that, in the β cell, caffeine increases intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) by a mechanism unrelated to its intracellular Ca^{2+}-mobilizing action (*5*). Furthermore, we used the patch-clamp technique to monitor the Ca^{2+}-sensitive K^+ conductance in β cells for detection of any small release of Ca^{2+} following the addition of cADP-ribose. This method is more sensitive than fluorimetric methods, and it has been used to record increases in $[Ca^{2+}]_i$ after intracellular application of IP_3 and guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) in the pancreatic β cell (*6*). Even so, we were unable to obtain evidence of Ca^{2+} release from intracellular stores after the addition of cADP-ribose in 14 out of 14 cells, whereas formation of IP_3 potently induced release of Ca^{2+} (Fig. 2).

These results raise several questions. First, what might be the reason for the absence of IP_3-induced Ca^{2+} release as reported by Takasawa *et al.*? Their procedure of purification of microsomes might have adversely affected the IP_3-sensitive Ca^{2+} stores, which can be easily damaged during fractionation (*7*). Second, why was cADP-ribose–induced Ca^{2+} release seen in their preparation but not in ours? We do not have a definitive answer to this question. It is possible that cADP-ribose–induced Ca^{2+} release in the β cell is small in magnitude and requires rigorous experimental conditions to be detected. Alternatively, the source of Ca^{2+} released in the experiments of Takasawa *et al.* might be cells other than β cells. It should be recalled that the islets used by Takasawa *et al.* contain a large proportion of cells that are not β cells. We avoided this potential problem by using a tumor cell line and an almost pure preparation of normal β cells as well as by performing experiments on single mouse β cells.

A possible explanation for our negative results with cADP-ribose could be that our preparation of the compound was inactive. However, precautions were taken to ensure that this was not the case. By using cADP-ribose from different sources, who verified the activity of the substance in other cell systems, we guarded against the possibility that the lack of effect in our experimental system was not simply a result of an inactive batch of the compound. Moreover, cADP-ribose seems to be a stable compound (*8*). With the aim of taking a more physiological experimental approach (that is, using cells instead of isolated organelles), we deliberately did not exactly duplicate the experiments conducted by Takasawa *et al.* Hence, there remains a possibility that some experimental factors might have adversely affected the cADP-ribose–sensitive release mechanism in our system.

Takasawa *et al.* also demonstrate that extracts of islets incubated in a high con-

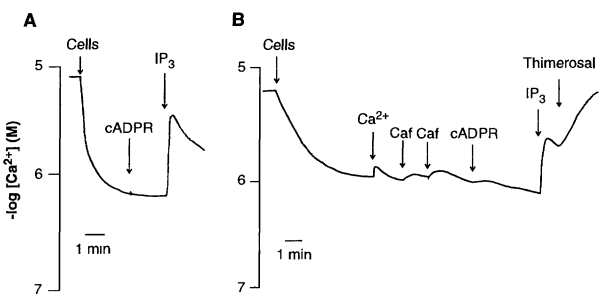

Fig. 1. Effect of cADP-ribose and IP_3 on electropermeabilized RINmF5 cells (**A**) and pancreatic β cells from *ob/ob* mice (**B**) as indicated by the release of Ca^{2+}. Pancreatic islets from fasting adult obese (*ob/ob*) mice were isolated by collagenase digestion and dispersed into small cell clusters by shaking in a Ca^{2+}- and Mg^{2+}-deficient medium (*11*). Electropermeabilized insulin-secreting cells were incubated in an intracellular-like buffer supplemented with adenosine triphosphate (ATP), an ATP-regenerating system, and the mitochondrial inhibitors antimycin and oligomycin. Changes in $[Ca^{2+}]$ were measured with a Ca^{2+}-sensitive minielectrode (*12*). Arrows indicate additions of cADPR (1 μM), $CaCl_2$ (1 nmol), IP_3 (5 μM), caffeine (4 mM), and thimerosal (50 μM). The effects of cADP-ribose were tested in three experiments with RINm5F cells and in three with pancreatic β cells from *ob/ob* mice.

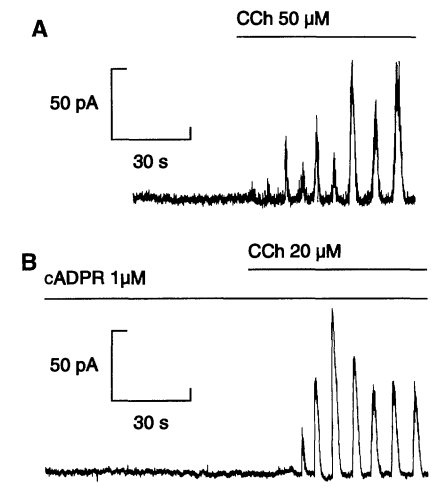

Fig. 2. Effect of cADP-ribose and carbachol (CCh) on pancreatic β cells from *ob/ob* mice as indicated by Ca^{2+}-activated K^+-currents (K_{Ca} currents). Membrane currents were recorded from single pancreatic β cells (*11*), with the use of the whole-cell configuration of the patch-clamp technique. (**A**) Oscillations in membrane K_{Ca} currents in response to CCh (50 μM) are pronounced. (**B**) Inclusion of cADP-ribose (1 μM) in the pipette solution resulted in no detectable effect . Addition of CCh (20 μM) to the same cells induced fluctuations in K_{Ca} currents. When the concentration of cADP-ribose was increased to 100 μM, there was still no effect on membrane currents (not shown).

centration of glucose release Ca^{2+} from islet microsomes and abolish Ca^{2+} release by the subsequent addition of cADP-ribose. This they attribute to the glucose-induced formation of cADP-ribose, which could not be measured. Glucose-induced arachidonic acid formation may mediate such a Ca^{2+} release (*9*). When multiple Ca^{2+}-mobilizing second messengers release Ca^{2+} from a common pool, the results can mistakenly be interpreted as "cross-desensitization." In a Perspective in the same issue, Antony Galione states that "glucose induces a rise in [cADP-ribose] concentrations in pancreatic β cell" (*10*). We know of no data to substantiate such a statement.

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13. We are grateful to H. C. Lee (University of Minnesota), T. F. Walseth (University of Minnesota), and A. Galione (Oxford University) for the generous supply of cADP-ribose.

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Response: We read with great interest the comment of Islam *et al.* They "deliberately did not exactly duplicate [our] experiments," and their work produced different results. It is as if we had said "Apples are sweet," and they responded, "No, you are wrong. Lemons are sour." Single cells do not behave like groups of cells, especially in the case of pancreatic β cells, where the interaction between a variety of subpopulations contributes greatly to insulin secretion (*1*). We demonstrated a correlation between cyclic ADP–ribose and insulin secretion (*2*); Islam *et al.* did not. We used glucose-sensitive rat islets, but Islam *et al.* used RINm5F cells and dispersed-cultured *ob/ob* mouse islet cells, which have negligible sensitivity to glucose (*1, 3, 4*). Islam *et al.* describe these cells as "insulin-secreting," but they actually secrete very little insulin, and no correlation was found between IP_3 and glucose-induced secretion.

Admittedly, the model we have been developing is still controversial (Fig. 1). However, it does contain some features that should be especially interesting to diabetes researchers. (i) It is based on experiments where the cells that were used, unlike *ob/ob* single β cells and RINm5F cells, were sensitive to glucose (*2, 5*). (ii) It can explain the reduction of insulin secretion and synthesis by various β-cytotoxic agents such as streptozotocin and alloxan (*6*). (iii) It can explain the prevention of this reduction by the poly(ADP-ribose) synthetase inhibitors nicotinamide and 3-aminobenzamide (*6*). (iv) It can explain the regulation of cyclic ADP–ribose by the synthesis and hydrolysis activity (*7*) of CD38 (*8*). This surface antigen was found in all tissues that we examined, but was especially prominent in high-glucose utilizing tissues such as islets, insulinomas, liver, kidney, brain, and pancreas (*8*).

In conclusion we believe that caution should be exercised when comparing the results of experiments that use single cells (and established cell lines) with those that use intact islets (*5*).

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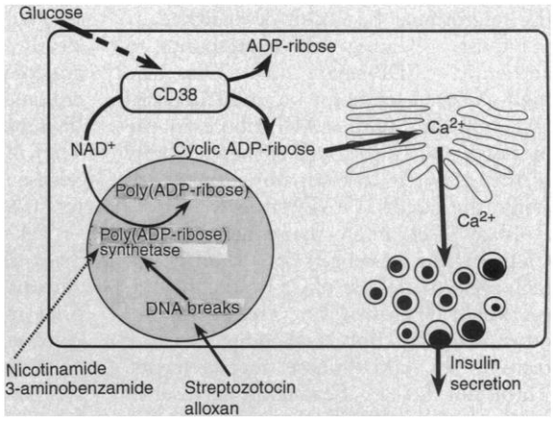

Fig. 1. A model of cyclic ADP–ribose mobilizing intracellular Ca^{2+} to induce islet β cells to secrete insulin. Cyclic ADP–ribose is generated from NAD^+ in glucose-stimulated β cells by CD38 (*8*). Cyclic ADP–ribose mobilizes Ca^{2+} from β cell endoplasmic reticulum to secrete insulin. Streptozotocin and other β cytotoxins cause DNA strand breaks that activate nuclear poly(ADP-ribose) synthetase, leading to NAD^+ depletion (*5*) and the inhibition of the cyclic ADP–ribose–mediated Ca^{2+} mobilization. Poly(ADP-ribose) synthetase inhibitors such as nicotinamide and 3-aminobenzamide prevent the NAD^+ depletion and, therefore, the glucose-induced insulin secreting process continues to function. β cells that cannot mobilize Ca^{2+} by cyclic ADP–ribose are unable to secrete insulin in response to glucose stimulation.

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9. We are grateful to B. Bell for provocative discussions.

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Response: Islam *et al.* state that they know of no data to substantiate my statement that "glucose induces a rise in [cADP-ribose] concentrations in pancreatic β cells" (*1*). In so doing, they seem to undervalue the specificity and usefulness of a cADP-ribose bioassay that has been used not only to demonstrate the presence of cADP-ribose in cells, but also to quantify cADP-ribose in mammalian tissues (*2*).

In their report, Takesawa *et al.* (*3*) demonstrated that treatment of rat pancreatic islets with a high glucose concentration resulted in the appearance in islet extracts of a factor which, when added to microsomes derived from both rat β cells and cerebellum, released Ca. They suggested that the high-glucose islet factor was cADP-ribose because, in both types of microsome, Ca^{2+} release by extracts from glucose-treated islets was not apparent if

the microsomes had been desensitized by previously releasing. Ca in response to authentic cADP-ribose (3). Thus the high-glucose islet factor was indistinguishable from authentic cADP-ribose in the bioassay, and on the basis of the specificity of this assay, it was not unreasonable to assign the factor as cADP-ribose.

Islam *et al.* imply that when multiple Ca-mobilizing messengers operate on a common pool, the release of Ca by one messenger would desensitize Ca^{2+} release by the other messenger, thus invalidating use of the bioassay for cADP-ribose measurements. This is not the case. Sea urchin microsomes exhibit both cADP-ribose and IP_3-sensitive Ca release, and these agents appear to come from a common pool as their release is nonadditive (4). Desensitization by either agent is homologous; that is, release by one messenger desensitizes further release by itself, but not by the other molecule. This is clearly the case, too, in cerebellar microsomes (3). Cerebellar and brain microsomes contain both cADP-ribose and IP_3-sensitive Ca release mechanisms (3, 5). They remain fully sensitive to IP_3 after Ca release induced by cADP-ribose or islet factors (3).

Takesawa *et al.* (3) go one step further. They show that drugs which lower the amount of nicotinamide adenine dinucleotide (NAD^+) in islets block the ability of glucose to produce the islet factor. Because NAD is the precursor for the enzymatic synthesis of cADP-ribose (6), these data taken together provide good evidence that glucose does elevate the amount of cADP-ribose in β cells, probably by stimulating its production from NAD.

The current high interest in cADP-ribose as a possible new second messenger (7) should provide the impetus for developing more sensitive and direct assays (8) for measuring concentrations of this molecule.

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