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Pancreatic β-Cells from Obese-Hyperglycemic Mice Are Characterized by Excessive Firing of Cytoplasmic Ca^{2+} Transients

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Pancreatic β-cells from obese-hyperglycemic (*ob/ob*) mice are widely used for studying the mechanisms of insulin release, including its regulation by the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). In this study, we compared changes of $[Ca^{2+}]_i$ in single β-cells isolated from *ob/ob* mice with those from lean mice using dual-wavelength microfluorometry and the indicator fura-2. There were no differences in the frequency, amplitude, and half-width of the slow oscillations induced by glucose. Most β-cells from the obese mice responded to 10 m*M* caffeine with transformation of the oscillations into sustained elevation of $[Ca^{2+}]_i$, a process counteracted by ryanodine. The β-cells from the obese mice were characterized by ample generation of $[Ca^{2+}]_i$ transients, which increased in number in the presence of glucagon. The transients became less frequent when leptin was added at a concentration as low as 1 n*M*. It is suggested that the excessive firing of $[Ca^{2+}]_i$ transients in the *ob/ob* mice is owing to the absence of leptin and is mediated by activation of the phospholipase C signaling pathway.

Key Words: Caffeine; Ca^{2+} transients; glucagon; leptin; ob/ob mice; pancreatic β-cells.

Introduction

Pancreatic islets from the obese-hyperglycemic (*ob/ob*) mice have been extensively used for studying insulin-releasing mechanisms because they are large and have a high proportion of β-cells *(1,2)*. It has been proposed that an increase in the number of β-cells within the islets represents the normal adaptation to hyperglycemia *(3)*. Although islets from *ob/ob* mice respond adequately to various insulin secretagogues, including glucose *(4,5)*, they are hypersensitive to stimuli activating the phospholipase C (PLC) signaling pathway *(6–8)*. Studies of glucose-stimulated individual islets *(9)* have indicated a regular pattern of pulsatile insulin release evoked by slow oscillations of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$).

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Pancreatic islets from *ob/ob* mice are deficient in glucagon-producing α-cells *(1)*, which are assumed to be involved in the intraislet regulation of the β-cell function *(10)*. It is therefore important to extend the analyses of the mechanisms for insulin release in the obese-hyperglycemic syndrome to isolated β-cells. Using dual-wavelength microfluorometry for measuring $[Ca^{2+}]_i$, it has been found that the β-cell has an intrinsic ability to oscillate *(11,12)* and generate rapid transients of $[Ca^{2+}]_i$ *(13,14)*. By comparing the increase in $[Ca^{2+}]_i$ in response to glucose with that in β-cells of lean mice, we now show that the β-cells from the obese mice have a similar oscillatory activity but a much more frequent firing of $[Ca^{2+}]_i$ transients, which are suppressed by leptin.

Results

Glucose induced slow oscillations of cytoplasmic Ca^{2+} in β-cells isolated from lean and obese mice (Fig. 1). There were no differences in the frequency, amplitude, and half-width when comparing the oscillations in the two types of mice (Table 1). The oscillations usually persisted in the presence of glucagon (see legend to Fig. 1). After the addition of 10 m*M* caffeine, the oscillations continued in a majority of the β-cells from the lean mice but were usually transformed into a sustained increase in $[Ca^{2+}]_i$ in β-cells from obese mice (Fig. 2). A χ^2 test confirmed that the oscillations were particularly sensitive to caffeine in β-cells from obese mice ($p < 0.05$). The glucose-induced oscillations remained unaffected when β-cells from lean (data not shown) and obese mice (Fig. 3, top) were exposed to 20 μ*M* ryanodine. Indeed, the addition of ryanodine usually reestablished the oscillatory activity in a medium containing caffeine (Fig. 3, middle and bottom).

The glucose-induced oscillations were sometimes superimposed with transients of $[Ca^{2+}]_i$. The transients were preferably seen in β-cells from obese mice exposed to glucagon (Fig. 1, bottom). Blockage of the voltage-dependent Ca^{2+} entry with methoxyverapamil made it possible to study the transients without disturbance from the slow oscillations of $[Ca^{2+}]_i$. Using this approach, it was found that the $[Ca^{2+}]_i$ transients were often synchronized even in β-cells lacking contact (Fig. 4). Table 2 indicates the frequency of $[Ca^{2+}]_i$ transients recorded under various conditions in β-cells exposed to methoxyverapamil. The β-cells from the *ob/ob* mice

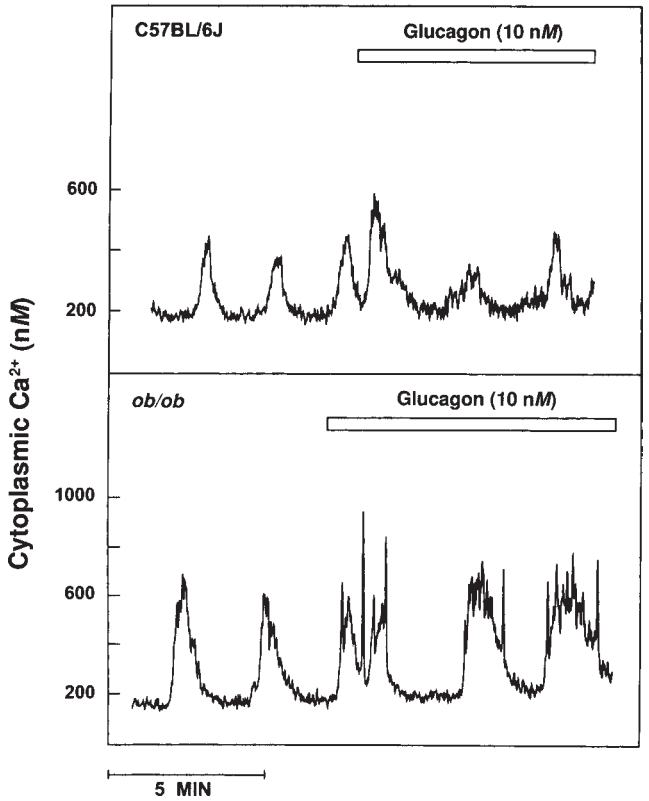

Fig. 1. Effects of glucagon on the β-cell oscillations of $[Ca^{2+}]_i$ induced by 11 m*M* glucose in lean (C57BL/6J) and obese (*ob/ob*) mice. The oscillations remained in the presence of glucagon in 9 of 10 experiments in the lean mice and in 8 of 10 experiments in the obese mice. How the exposure to glucagon results in transients of $[Ca^{2+}]_i$ superimposed on the oscillations in the *ob/ob* mouse is seen.

Table 1
Characteristics of Glucose-Induced Slow Oscillations of $[Ca^{2+}]_i$ in β-Cells from Lean (C57BL/6J) and Obese (*ob/ob*) Mice[a]

Characteristic	Lean (23)	Obese (12)
Frequency (oscillations/min)	0.31 ± 0.02	0.30 ± 0.03
Amplitude (n*M*)	350 ± 25	370 ± 27
Half-width (min)	1.3 ± 0.1	1.0 ± 0.1

[a]The oscillations were induced by 11 m*M* glucose in individual β-cells. Values are mean ± SEM. The number of experiments is given in parentheses.

were more active in generating $[Ca^{2+}]_i$ transients than those from the lean NMRI and C57BL/6J mice both in the control situation and in the presence of glucagon, caffeine, carbachol, or ryanodine. The β-cell firing of $[Ca^{2+}]_i$ transients was low in the *ob*/+ heterozygotes in comparison with that in the *ob/ob* mice.

Since the *ob/ob* mice lack leptin, we tested whether short-term exposure to this hormone affected the excessive firing of $[Ca^{2+}]_i$ transients seen in the β-cells from these mice. This was found to be the case (Fig. 5). As shown in Table 3, leptin had a suppressive action on the generation of the transients at a concentration as low as 1 n*M*.

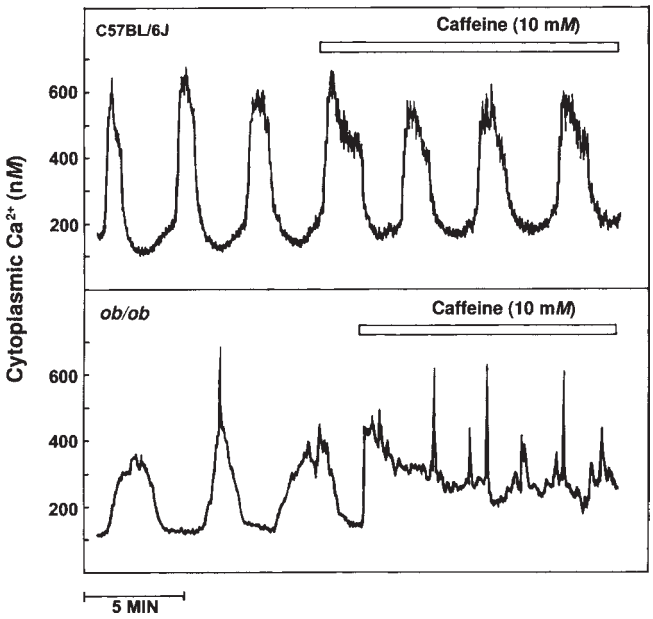

Fig. 2. Effects of caffeine on the β-cell oscillations of $[Ca^{2+}]_i$ induced by 11 m*M* glucose in lean (C57BL/6J) and obese (*ob/ob*) mice. After the addition of 10 m*M* caffeine, the oscillations were usually transformed into sustained elevation of $[Ca^{2+}]_i$ in the obese mice (7 of 8 experiments) but not in the lean mice (4 of 14 experiments).

Discussion

Islets from *ob/ob* mice with the genetic background C57BL have been used extensively for investigating the insulin-releasing mechanisms, since they provide large amounts of β-cells with little contribution of other types of cells *(1,3)*. Despite the deficiency of glucagon-producing α-cells, the islets from the *ob/ob* mice respond appropriately to a wide variety of secretory stimuli, including glucose *(4,5)*. A characteristic feature of isolated β-cells is the slow oscillations of $[Ca^{2+}]_i$ induced by glucose. The ability to oscillate is a sensitive indicator of the well-being of the β-cell, and minor damage is known to result in disappearance of the $[Ca^{2+}]_i$ rhythmicity *(15,16)*. We now demonstrate that β-cells from lean and obese mice generate $[Ca^{2+}]_i$ oscillations with similar frequency, amplitude, and half-width.

Previous studies in our laboratory have indicated that the oscillations are transformed into a sustained increase of $[Ca^{2+}]_i$ when the β-cells are depolarized with glycine, alanine, or arginine *(17)* or the cyclic adenosine monophosphate (cAMP) content is raised with glucagon or theophylline *(18,19)*. By performing the experiments with less depolarized β-cells (4.0 instead of 5.9 m*M* K^+), we observed that the oscillations persist in most β-cells after the addition of glucagon. The observation that 10 m*M* caffeine transforms the oscillations into sustained elevation is consistent with

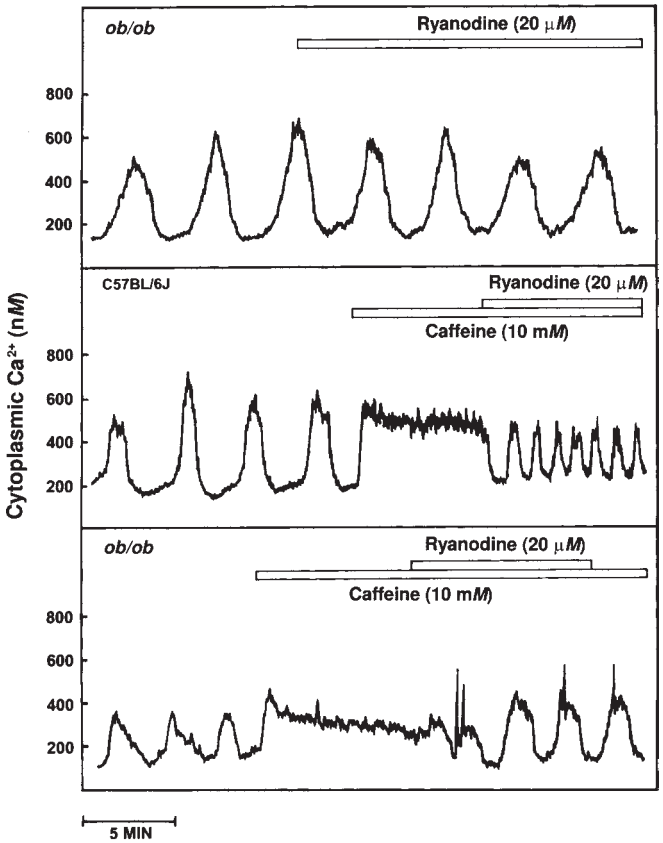

Fig. 3. Effects of ryanodine in the presence of 11 m*M* glucose on $[Ca^{2+}]_i$ in β-cells from lean (C57BL/6J) and obese (*ob/ob*) mice. Ryanodine was without effect on the oscillations of $[Ca^{2+}]_i$ in both the lean (all of five experiments) and obese (all of five experiments) mice. In β-cells responding with sustained elevation to caffeine, the oscillations were restored by ryanodine in four of five experiments with obese mice and in a single experiment performed with a lean mouse.

the report *(20)* that this compound has effects on the Ca^{2+} entry into the β-cells in addition to those mediated by an increase in cAMP.

Glucose has been reported to induce a rise in cyclic adenosine 5'-diphosphate ribose (cADPR) in pancreatic islets *(21)*, probably by altering the ADP-ribosyl cyclase/cADPR hydrolase ratio of the ectoenzyme C38 *(22)*. Autoantibodies against C38 have been detected in patients with type 2 diabetes and found to inhibit glucose-stimulated insulin release from isolated islets *(23)*. Although there is evidence for the presence of the type 2 isoform of the ryanodine receptor in β-cells from lean *(21)* and obese mice *(24)*, doubts have been expressed regarding a messenger role for cADPR in coupling the metabolism of glucose to the release of insulin *(25,26)*. We now observe that ryanodine does not affect the β-cell oscillations of $[Ca^{2+}]_i$ responsible for the glucose-induced pulses of insulin release. However, the addition of ryanodine sometimes restored the oscillatory activity in β-cells responding to glucose with a sustained increase in $[Ca^{2+}]_i$ in the presence of caffeine. The observation that ryanodine counteracts effects on $[Ca^{2+}]_i$ induced by caffeine is not unique to pancreatic β-cells but has also been observed in smooth muscle *(27)* and adrenal chromaffin *(28)* cells.

In addition to inducing oscillations of $[Ca^{2+}]_i$ *(12)*, glucose promotes rapid transients owing to intracellular mobilization of the ion *(13)*. The transients are usually superimposed on the slow $[Ca^{2+}]_i$ oscillations *(13,14)*. However, in the present study the transients were recorded without the background disturbance of the slow oscillations by blocking the voltage-dependent Ca^{2+} channels with methoxyverapamil. Using this protocol, it was possible to confirm previous observations that isolated β-cells from *ob/ob* mice generate transients of $[Ca^{2+}]_i$, the frequency of which increases considerably in the presence of glucagon or other agents raising cAMP *(13)*. A pertinent feature of these transients is that they often appear in synchrony in β-cells lacking contact *(14)*. We now demonstrate the presence of synchronized transients also in β-cells from lean mice including *ob*/+ heterozygotes. However, the frequency of these transients was very low compared with that seen in the β-cells of the *ob/ob* mice, irrespective of whether the medium was supplemented with glucagon, caffeine, or carbachol.

Whereas the slow oscillations are known to reflect a periodic entry of Ca^{2+} into the β-cells *(12)*, the transients can be attributed to mobilization of Ca^{2+} from the endoplasmic reticulum *(13)*. It has been proposed that the signal for mobilizing the β-cell stores of Ca^{2+} in *ob/ob* mice differs from that in lean C57BL/6J mice in being mediated by inositol triphosphate (IP_3) instead of cADPR *(21)*. However, recent studies in our laboratory have revealed that IP_3 mobilizes most of the thapsigargin-sensitive Ca^{2+} from permeabilized islets also in the lean mouse *(26)*. Ryanodine, caffeine, and cADPR lacked Ca^{2+}-mobilizing effects when testing the possibility *(24,29)* that activation of the ryanodine receptor requires cAMP-dependent phosphorylation. It is possible that our observation of an excessive firing of $[Ca^{2+}]_i$ transients in the β-cells from *ob/ob* mice is somehow related to an increased activity of PLC. It is well established that the islets from the *ob/ob* mice have an exaggerated insulin release in response to cholinergic stimuli *(6–8)*.

Being sufficiently pronounced to temporarily interrupt the entry of Ca^{2+} by activating a hyperpolarizing K^+ current *(30)*, the transients have been proposed to coordinate the $[Ca^{2+}]_i$ rhythmicity responsible for the pulsatile release of insulin *(31)*. The physiologic relevance of an excessive generation of $[Ca^{2+}]_i$ transients in β-cells from *ob/ob* mice is difficult to evaluate. In the in vivo situation it can be expected that these β-cells are not only exposed to raised concentrations of glucose but also to low levels of glucagon owing to a reduced islet proportion of α-cells *(1)*. An increased frequency of irregularly occurring transients may not only increase the coupling force for the synchronization of the β-cell oscillations of $[Ca^{2+}]_i$ but also aid the release of insulin by initiating brief periods of accelerated exocytosis.

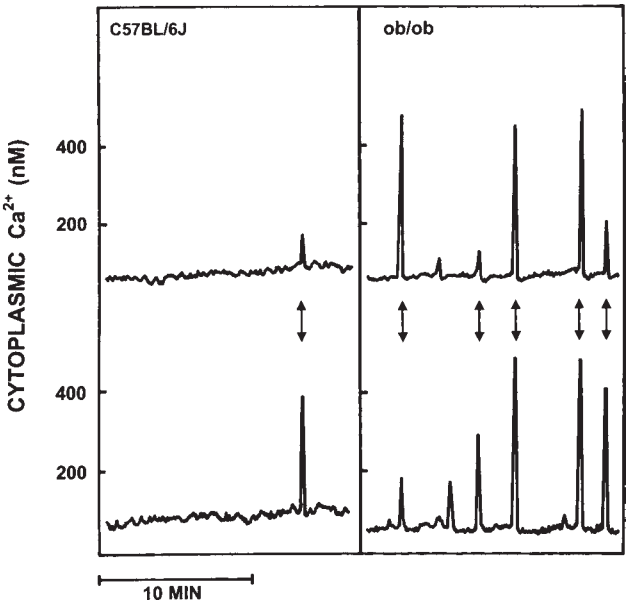

Fig. 4. Transients of $[Ca^{2+}]_i$ in β-cells from lean (C57BL/6J) and obese (*ob/ob*) mice exposed to a medium containing 20 m*M* glucose, 20 n*M* glucagon, and 50 μ*M* methoxyverapamil. Arrows indicate synchronization of spontaneously occurring transients in two cells separated by a distance of 22 (lean mouse) and 24 μm (obese mouse), respectively.

Table 2
Frequency of $[Ca^{2+}]_i$ Transients in Isolated β-Cells from Lean (NMRI, C57BL/6J, and *ob*/+) and Obese Mice[a]

Animal	Additives	Transients/10 min
NMRI	Glucagon (20 n*M*)	0.04 ± 0.02 (10)
NMRI	Caffeine (2 m*M*)	0.09 ± 0.09 (5)
NMRI	Caffeine (20 m*M*)	0.05 ± 0.14 (5)
C57BL/6J	Glucagon (20 n*M*)	0.10 ± 0.03 (24)
C57BL/6J	Glucagon (20 n*M*) + carbachol (100 n*M*)	0.07 ± 0.05 (8)
ob/+	Glucagon (20 n*M*)	0.14 ± 0.03 (6)
ob/ob	—	0.16 ± 0.04 (17)
ob/ob	Glucagon (20 n*M*)	1.97 ± 0.38 (18)
ob/ob	Caffeine (2 m*M*)	1.06 ± 0.25 (9)
ob/ob	Caffeine (20 m*M*)	0.85 ± 0.14 (11)
ob/ob	Caffeine (2 m*M*) + ryanodine (5 μ*M*)	1.06 ± 0.41 (7)
ob/ob	Glucagon (20 n*M*) + carbachol (100 n*M*)	2.90 ± 0.50 (16)

[a]The experiments were performed in the presence of 20 m*M* glucose and 50 μ*M* methoxyverapamil. The number of experiments is given in parentheses. Values are mean ± SEM.

The primary cause of the obesity in *ob/ob* mice is a lack of leptin owing to a mutation in the *ob* gene *(32)*. Leptin is known to modulate food intake and energy metabolism by activating a receptor in the hypothalamus. However, leptin may also have direct physiologic functions outside of the central nervous system. Both the islets from lean and *ob/ob* mice are known to express the full-length leptin receptor proposed to participate in signal transduction *(33)*. The exaggerated insulin secretory response to muscarinic receptor agonists in *ob/ob* mice is thought to be mediated by a PLC-activated pathway, which is suppressed by leptin in lean mice *(6–8)*. The present study provides additional arguments for a direct leptin interaction with pancreatic β-cells by demonstrating that this hormone suppresses the firing of $[Ca^{2+}]_i$ transients. The leptin effect was established already at 1 n*M*, a concentration close to that seen in circulating blood of lean rodents *(34)*. The leptin suppression of the $[Ca^{2+}]_i$ transients may not only be owing to a reduced PLC activity but also to an interference with the cAMP sensitization of the IP_3 receptor *(13)*. In rat insulinoma cells, leptin inhibits cAMP-induced release of insulin without affecting the secretory response to PLC activation *(35)*. However, it has also been reported that leptin constrains the PLC-mediated insulin secretion in islets from *ob/ob* mice without affecting the secretory response to glucagon-like peptide-1 *(8)*.

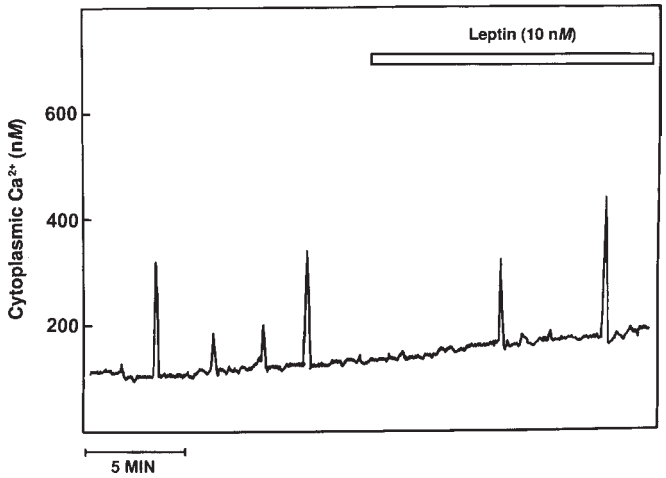

Fig. 5. Transients of $[Ca^{2+}]_i$ in a β-cell from an obese (*ob/ob*) mouse before and after the addition of 10 n*M* leptin to a medium containing 20 m*M* glucose, 20 n*M* glucagon, and 50 μ*M* methoxyverapamil.

Table 3
Frequency of $[Ca^{2+}]_i$ Transients in Isolated β-Cells from Obese Mice Before and After Addition of Leptin[a]

	Transients/10 min		
Leptin (n*M*)	Control period	Test period	Effect of leptin
1	3.61 ± 0.48	3.08 ± 0.41	–0.53 ± 0.20** (15)
10	4.10 ± 0.70	3.17 ± 0.70	–0.94 ± 0.29*** (11)
100	4.41 ± 0.53	3.37 ± 0.61	–1.04 ± 0.38* (7)

[a]The experiments were performed in the presence of 20 m*M* glucose, 20 n*M* glucagon, and 50 μ*M* methoxyverapamil. The number of experiments is given in parentheses. Values are mean ± SEM. *$p < 0.05$; **$p < 0.025$; ***$p < 0.01$.

In summary, we have shown that glucose-stimulated β-cells isolated from *ob/ob* mice have regular $[Ca^{2+}]_i$ oscillations combined with an excessive firing of transients. It is suggested that the excessive firing of $[Ca^{2+}]_i$ transients in the *ob/ob* mice is owing to the absence of leptin and is mediated by activation of the PLC signaling pathway.

Materials and Methods

Chemicals

Reagents of analytical grade and deionized water were used. Collagenase and bovine serum albumin (BSA) (fraction V) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany), and fetal calf serum (FCS) was obtained from Gibco (Paisley, UK). Carbachol, ryanodine, glucagon, caffeine, and leptin (mouse recombinant) were from of Sigma (St. Louis, MO), and fura-2 acetoxymethyl ester was provided by Molecular Probes (Eugene, OR). Methoxyverapamil was donated by Knoll A.G. (Ludwigshafen am Rhein, Germany).

Preparation of β-Cells

Islets of Langerhans were isolated with the aid of collagenase from the splenic part of the pancreas from 5- to 13-mo-old lean mice (NMRI and C57BL/6J) as well as from noninbred *ob/ob* and *ob*/+ mice. The latter animals were 10–14 mo old and taken from a local colony established 40 yr ago from breeding couples obtained from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, ME *(1)*. Single cells were prepared by shaking the islets in a Ca^{2+}-deficient medium *(36)*. After suspension in RPMI 1640 medium supplemented with 10% (v/v) FCS, 100 IU/mL of penicillin, 100 µg/mL of streptomycin, and 30 µg/mL of gentamicin, the cells were allowed to attach to circular 25-mm cover slips during culture for 1–4 d at 37°C in an atmosphere of 5% CO_2.

Loading of β-Cells with Fura-2

After culture, the subsequent experimental handling was performed with a basal medium containing 0.5 mg/mL of BSA and 125 m*M* NaCl , 4 m*M* KCl , 1.2 m*M* $MgCl_2$, 1.3 m*M* (photomultiplier recordings) or 2.6 m*M* (image analysis) $CaCl_2$, and 25 m*M* HEPES, with pH adjusted to 7.40 with NaOH. After rinsing, the cells were loaded with 0.5 µ*M* fura-2 acetoxymethyl ester for 30–40 min of incubation at 37°C. The cover slips with the attached cells were then washed and used as exchangeable bottoms of an open chamber containing 160 µL of medium *(37)*. The chamber was placed on the stage of an inverted microscope within a climate box (37°C), and the cells were superfused at a rate of 0.75 mL/min. The selection of β-cells for analysis was based on their large size and low nuclear/cytoplasmic ratio compared with the islet cells secreting glucagon and somatostatin *(38)*.

Photomultiplier Recordings

After loading with fura-2, $[Ca^{2+}]_i$ was measured according to the principles of Grynkiewicz et al. *(39)*, assuming a K_d for the Ca^{2+} fura-2 complex of 224 n*M*. A filter changer of a time-sharing multichannel spectrophotometer *(40)* provided excitation light flashes of 1-ms duration every 10 ms at 340 and 380 nm, and the emission was recorded with a photomultiplier.

Image Analyses

Images of fura-2-loaded cells were collected at 510 nm with an intensified charge-coupled device camera after dual-wavelength excitation *(41)*. Pairs of 340- and 380-nm images, consisting of 16 accumulated video frames divided by 8, were captured during 2.8 s followed by a 4-s delay. Ratio frames were calculated after background subtraction, and $[Ca^{2+}]_i$ was estimated according to the principles of Grynkiewicz et al. *(39)*.

Statistical Evaluation

In the photomultiplier recordings, each experiment refers to analyses of individual β-cells on separate cover slips. In the image analyses, each experiment refers to the average number of transients (>50 n*M*) found in 4–12 cells from the same cover slip. Transients occurring within three successive ratio frames were considered synchronized. A given protocol was tested with cells from at least five animals. Statistical analyses were performed using the χ^2 test with Yates correction and Student's *t*-test for paired data. Results are presented as mean values ±SEM.

Acknowledgments

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