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# Repetitive mitochondrial $Ca^{2+}$ signals synchronize with cytosolic $Ca^{2+}$ oscillations in the pancreatic beta-cell line, MIN6

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**Summary** We examined the relationship between cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) and mitochondrial matrix $Ca^{2+}$ concentration ($[Ca^{2+}]_m$) in the pancreatic beta-cell line, MIN6. $[Ca^{2+}]_c$ was monitored in a single or a group (30 cells) of fura-2-loaded MIN6 cells, and $[Ca^{2+}]_m$ was measured in a group ($1 \times 10^6$ cells) of MIN6 cells stably transfected with aequorin targeted at the mitochondria. Exogenous ATP (0.25 mmol/l) produced a single transient increase in $[Ca^{2+}]_c$ whereas 22 mmol/l KCl produced a sustained plateau increase. ATP and KCl evoked transient increases in $[Ca^{2+}]_m$ but with distinct time courses of $[Ca^{2+}]_m$ decline: the $[Ca^{2+}]_m$ increase induced by ATP decreased more rapidly than that induced by KCl. Nitrendipine (3 μmol/l), a blocker of L-type $Ca^{2+}$ channels, inhibited both $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ signals in response to KCl and tolbutamide, but not those to ATP. Peak levels of $[Ca^{2+}]_m$ increase (around 2 μmol/l) exceeded those of $[Ca^{2+}]_c$ increase (around 500 nmol/l). A rise in glucose concentration from 3 to 30 mmol/l induced oscillations of $[Ca^{2+}]_c$ that overlay the sustained increases in $[Ca^{2+}]_c$ in single cells. An oscillatory increase in $[Ca^{2+}]_m$ was similarly observed in response to glucose. Addition of 10 mmol/l 2-ketoisocaproic acid at 20 mmol/l glucose further increased the plateau level of $[Ca^{2+}]_c$ and the frequency of $[Ca^{2+}]_c$ oscillations, which were correlated with a further increase in $[Ca^{2+}]_m$. In response to pulsatile exposure to KCl, $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ increased synchronously. These data suggest that an oscillatory increase in $[Ca^{2+}]_m$ in beta cells, the signal which is thought to be necessary for continuous stimulation of mitochondrial metabolism, is produced synchronously with the $[Ca^{2+}]_c$ oscillations. [Diabetologia (1998) 41: 279–286]

**Keywords** Pancreatic beta-cell line, mitochondrial calcium, cytosolic calcium, oscillation, aequorin, insulin secretagogue.

Pancreatic beta cells are unique among hormone-secreting cells in that their metabolism of glucose and other nutrients plays a dominant role in stimulus-secretion coupling [1, 2]. Metabolic signalling molecules, such as adenine and guanine nucleotides, have several functions, including the inhibition of ATP-sensitive potassium channels that trigger increases in the electrical activity of beta cells [3] and provision of energy for exocytosis of insulin [4, 5]. The production of such high-energy compounds in pancreatic beta cells is thought to involve mitochondrial oxidative phosphorylation [reviewed in 6, 7]. In fact, MIN6 cells depleted of mitochondrial DNA do not exhibit glucose-induced insulin secretion as a result of loss of mitochondrial respiratory function [8].

The increase in $[Ca^{2+}]_m$ is important for mitochondrial fuel metabolism because several key enzymes in the mitochondrial matrix and inner membrane, including the pyruvate and 2-oxoglutarate dehydrogenase complexes, NAD-isocitrate dehydrogenase, mitochondrial glycerol phosphate dehydrogenase and in-

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*Abbreviations:* $[Ca^{2+}]_c$, Cytosolic $Ca^{2+}$ concentration; $[Ca^{2+}]_m$, mitochondrial $Ca^{2+}$ concentration; KIC, 2-ketoisocaproic acid; DMEM, Dulbecco's modified Eagle's medium; KRB, Krebs-Ringer bicarbonate buffer; BSA, bovine serum albumin; FCCP, p-trifluoromethoxyphenylhydrasone; $\tau$, time constant.

organic pyrophosphatase, are regulated by $Ca^{2+}$ at physiological concentrations ranging from nanomolar to micromolar [9–12]. A study has indicated that the level of NAD(P)H, a product of these enzymes, increases and oscillates in response to glucose [13], raising the possibility that $[Ca^{2+}]_m$ also oscillates in response to glucose. Further, a recent study has shown that glucose-induced insulin secretion coincides with the elevation of both $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ [14]. However, the precise relationship between $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ in beta cells remains obscure. It is important, therefore, to analyse dynamic changes in $[Ca^{2+}]_m$ in combination with $[Ca^{2+}]_c$ during exposure to glucose and other insulin secretagogues.

The pancreatic beta-cell line, MIN6, retains the ability to secrete insulin in response to physiological glucose concentrations [15]. Further, the characteristics of glucose transport and metabolism in MIN6 cells closely resemble those of isolated islets [16] and the increase in $[Ca^{2+}]_c$ depends on glucose metabolism [17]. Taken together, this cell line is a suitable model for studying the kinetics of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ induced by glucose or other secretagogues.

We transfected cDNA for the $Ca^{2+}$-sensitive photoprotein, aequorin, fused in-frame with cDNA encoding a mitochondrial presequence into MIN6 to measure $[Ca^{2+}]_m$ [18]. The $[Ca^{2+}]_m$ signals were then compared to the $[Ca^{2+}]_c$ signals measured with fura-2. We believe that the comparison is valid because the estimates of $[Ca^{2+}]_c$ made with the two probes, recombinant cytosolic aequorin and fura-2, were reported to be essentially similar, although the peak level of $[Ca^{2+}]_c$ is estimated to be lower with fura-2 due to its $Ca^{2+}$-buffering action [19].

## Materials and methods

*Cell culture and stable transfection with aequorin.* MIN6 cells were grown in Dulbecco's modified Eagle's medium (DMEM: 25 mmol/l glucose) equilibrated with 5 % $CO_2$ and 95 % air at 37 °C. The medium was supplemented with 15 % fetal calf serum, 50 mg/l streptomycin and 75 mg/l penicillin sulphate. The chimeric mitochondrial aequorin cDNA coding for the N-terminal presequence of cytochrome oxidase subunit VIII plus aequorin (Molecular Probes, Eugene, Ore., USA) was subcloned under the chicken beta-actin promoter of the expression vector pCXN [20], which contains a neomycin phosphotransferase II gene. The resulting expression vector was transfected into MIN6 cells using Lipofectin (Life Technologies Inc., Gaithersburg, MD, USA). Transfectant clones were established by isolating colonies resistant to 500 μg/ml of antibiotic G418 (Geneticin; Life Technologies, Inc.). After selection, independent cellular clones were isolated and expanded. Two clones were strongly positive by Northern analysis of aequorin expression and secreted insulin in response to physiological glucose concentrations. All experiments shown were performed with one ( # 3) of these clones.

*Measurement of $[Ca^{2+}]_c$.* $[Ca^{2+}]_c$ was measured by dual-wavelength fura-2 microfluorometry and video imaging [21]. MIN6 cell transfectants (seeding density $1 \times 10^6$/2 ml) were cultured on glass coverslips (33 mm diameter) for 4 days (giving almost full confluence) in DMEM plus 15 % fetal calf serum and antibiotics as mentioned above. Cells on coverslips were loaded with 2 μmol/l fura-2/acetoxymethilester (Molecular Probes) for 4 h at 4 °C in Krebs-Ringer bicarbonate buffer (in mmol/l: 129 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgCl_2$, 1.2 $KH_2PO_4$, 5.0 $NaHCO_3$, 10 HEPES-NaOH, pH 7.4; KRB) containing 0.1 % bovine serum albumin (BSA) with 3 mmol/l glucose. The cells were then mounted in a chamber (1.5 ml), placed on the stage of a TMD inverted microscope (Nikon, Tokyo, Japan), and superfused using a peristaltic pump (Watson Marlow, Falmouth, UK) at a rate of 3 ml/min at 37 °C with KRB containing 0.1 % BSA with basal (3 mmol/l glucose) or test solutions. The fura-2-loaded cells were excited at 340 nm and 380 nm alternately at 2.5 s intervals, the emission signals at 510 nm were detected by an intensified charge coupled device (ICCD) camera, and the ratio image was produced by an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan). The ratio was converted to $[Ca^{2+}]_c$ according to a calibration curve obtained from the relationship between free $Ca^{2+}$ concentration and the ratio determined in a cytosol-mimicking solution using Ca-EGTA buffer and fura-2 free acid.

*Measurement of aequorin luminescence.* MIN6 cell transfectants were cultured on glass coverslips as described. Functional aequorin was reconstituted in situ by 4 h incubation with 2.5 μmol/l coelenterazine (Molecular Probes) in the buffer solution and conditions as mentioned for fura-2. Luminescence was measured during perfusion (3.0 ml/min) with KRB containing 0.1 % BSA at 37 °C, plus additions as indicated. Emitted light was gathered through the coverslip using a high sensitivity photomultiplier positioned close (5 mm) to the cells in a custom-built chamber (1.5 ml). The photomultiplier output was collected (1 data point/1 s) using a P101 system (Nikon). After application of the stimuli, the cells were disrupted rapidly by perfusion with 200 μmol/l digitonin plus 10 mmol/l $CaCl_2$ in order to determine the total aequorin content. The background luminescence, measured from the same number of non-transfected cells preincubated with coelenterazine, was less than 1 % of the peak luminescence responses to KCl obtained in the transfected cells, and therefore it was neglected. Data were calibrated according to the equation for model B reported by Allen et al. [22]. We employed the values of parameters from data on recombinant aequorin [19].

*Data analysis.* All data are expressed as the mean ± SEM. Analysis was by Student's *t*-test, with $p$ less than 0.05 taken as statistically significant.

## Results

*Mitochondrial location of the targeted aequorin.* We tested the location of the mitochondrially targeted aequorin by comparing the increase of $[Ca^{2+}]_c$ and aequorin signal induced by 22 mmol/l KCl in the presence or absence of the mitochondrial uncoupler, p-trifluoromethoxyphenylhydrasone (FCCP) (Fig. 1 A). The increase of $[Ca^{2+}]_c$ induced by a high concentration of KCl in the presence or absence of 1 μmol/l FCCP was 947 ± 25 nmol/l and 978 ± 37 nmol/l ($n$ = 90, 30 cells in each experiment), respectively. These values did not differ significantly ($p = 0.76$). In con-

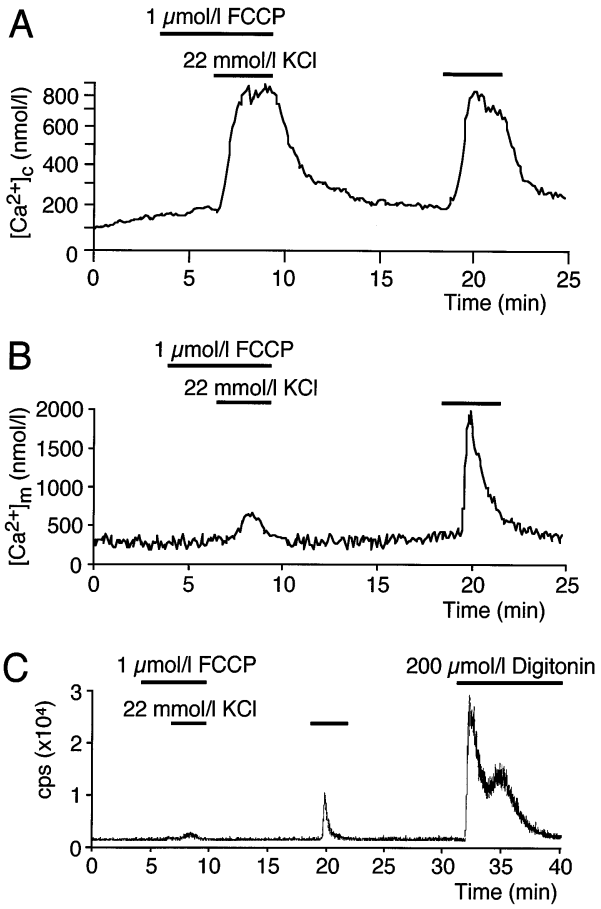


**Fig. 1 A–C.** Localization of aequorin targeted at the mitochondria in the stably transfected MIN6 cells. **A**: $[Ca^{2+}]_c$ recorded in a single fura-2-loaded MIN6 cell. Where indicated, 22 mmol/l KCl was applied in the presence and absence of 1 μmol/l FCCP. **B** and **C**: aequorin signals, measured in populations of MIN6 cells in monolayers with the same protocol as above, are expressed by the raw count per second (cps) value (**C**) or by the calculated $[Ca^{2+}]_m$ value (**B**). At the end of measurements, the cells were perifused with a solution of 10 mmol/l $CaCl_2$ plus 200 μmol/l digitonin in water to determine the total aequorin content as shown in (**C**). The trace represents 3 similar experiments. Glucose concentration was 3 mmol/l in (**A**) and (**B**)

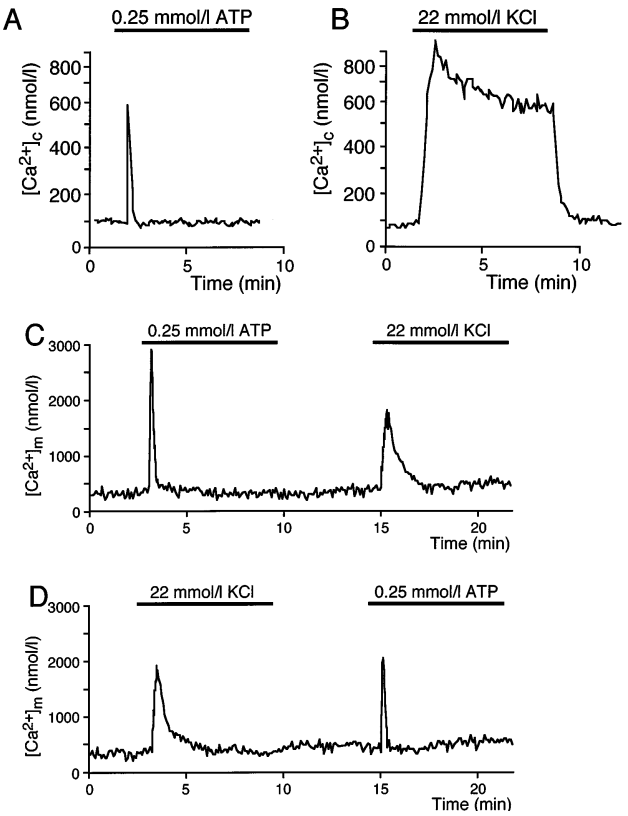


**Fig. 2 A–D.** Comparison of the effects of exogenous ATP and high KCl on $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$. $[Ca^{2+}]_c$ was recorded from 2 single fura-2-loaded MIN6 cells (**A** and **B**). $[Ca^{2+}]_m$ was measured in populations of MIN6 cells (**C** and **D**). The order of the two stimuli was reversed in **C** and **D**. The perfusion medium contained 3 mmol/l glucose with the following additions where indicated: 0.25 mmol/l ATP, 22 mmol/l KCl

trast, the increase in aequorin signal induced by KCl was attenuated when FCCP was present (Fig. 1 B, C). The uptake of $Ca^{2+}$ into the mitochondrial matrix is catalysed by an electrophoretic uniporter that is driven by the membrane potential component of the proton-motive gradient set up through proton extrusion by the respiratory chain. FCCP abolishes the mitochondrial inner membrane potential and thereby attenuates the driving force for mitochondrial $Ca^{2+}$ uptake. Our results indicate, therefore, the mitochondrial location of the targeted aequorin and validates monitoring the aequorin signal as $[Ca^{2+}]_m$ [14, 18, 23].

*Comparison of the effects of exogenous ATP, high KCl and tolbutamide on $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ in MIN6 cells.* When 0.25 mmol/l ATP was applied externally for 7 min, a single transient increase in $[Ca^{2+}]_c$ was observed above a steady basal $[Ca^{2+}]_c$ in about 80 % of the cells examined (Fig. 2 A). In contrast, 22 mmol/l KCl produced a sustained increase of $[Ca^{2+}]_c$ during the exposure in each cell (Fig. 2 B). The mean amplitude of the $[Ca^{2+}]_c$ response to ATP and KCl was 539 ± 22 nmol/l and 911 ± 23 nmol/l ($n$ = 120, 30 cells in each experiment), respectively. Increases of $[Ca^{2+}]_m$ induced by ATP and high KCl were similarly transient, though their time course of decrease differed (Fig. 2 C, D). Mean amplitudes of the $[Ca^{2+}]_m$ increases in response to ATP and KCl, 2663 ± 232 nmol/l ($n$ = 4) and 2148 ± 176 nmol/l ($n$ = 4) respectively, were much higher than those of $[Ca^{2+}]_c$. During stimulation with ATP, $[Ca^{2+}]_m$ returned to the pre-stimulation level within 1 min, a time course similar to that of $[Ca^{2+}]_c$ elicited by ATP. The time constant ($\tau$) for luminescence decrease was 0.25 ± 0.04 min ($n$ = 4). In the case of KCl stimulation, $[Ca^{2+}]_m$ decreased more slowly and $\tau$ was 1.04 ± 0.11 min ($n$ = 4). The difference in the time course of $[Ca^{2+}]_m$ reduction between ATP and KCl was ob-

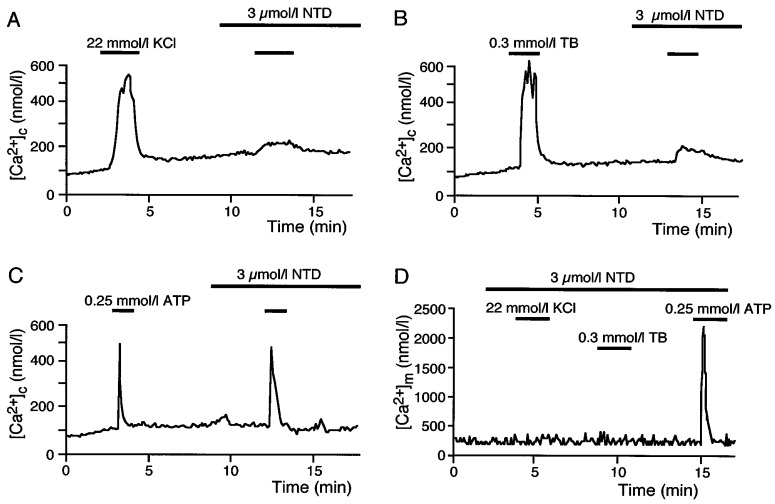


**Fig. 3 A–D.** Effects of nitrendipine (NTD), an L-type $Ca^{2+}$-channel blocker, on $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ increase induced by exogenous ATP, high KCl or tolbutamide. $[Ca^{2+}]_c$ was recorded from 3 single fura-2-loaded MIN6 cells (**A**–**C**). Where indicated, 22 mmol/l KCl, 0.3 mmol/l tolbutamide (TB) or 0.25 mmol/l ATP was applied in the presence and absence of 3 μmol/l nitrendipine. $[Ca^{2+}]_m$ was measured in populations of MIN6 cells in the presence of 3 μmol/l nitrendipine (**D**). The glucose concentration was 3 mmol/l in each experiment

served consistently and was independent of the sequence of adding the two stimuli (Fig. 2 C, D).

In the presence of 3 μmol/l nitrendipine, a blocker of the voltage-sensitive L-type $Ca^{2+}$ channel, the $[Ca^{2+}]_c$ increase induced by 22 mmol/l KCl and by 0.3 mmol/l tolbutamide was attenuated (Fig. 3 A, B) and the $[Ca^{2+}]_m$ increase in response to these two agents was completely inhibited (Fig. 3 D). These results were consistent with previous reports that KCl and tolbutamide increase $[Ca^{2+}]_c$ largely by stimulating $Ca^{2+}$ influx via L-type $Ca^{2+}$ channels [24, 25]. The present results suggest, therefore, that the $Ca^{2+}$ influx spread directly toward the mitochondrial matrix. We cannot, however, assert whether or not a small increase of $[Ca^{2+}]_c$ induced by high KCl or tolbutamide stimulation during the perfusion of nitrendipine observed in Figure 3 A and B was transmitted to the mitochondrial matrix because of the difficulty in detecting a small $[Ca^{2+}]_m$ transient due to the low accuracy of aequorin at $[Ca^{2+}]_m$ concentrations below 300 to 400 nmol/l [19, 22].

Interestingly, the effect of exogenously applied 0.25 mmol/l ATP on $[Ca^{2+}]_c$ was influenced little by the presence of nitrendipine (Fig. 3 C), and the $[Ca^{2+}]_m$ increase evoked by ATP in the presence of nitrendipine was similar in magnitude to that in the absence of the blocker (Figs. 2 C, 3 D).

*Effects of glucose and KIC on $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$.* When glucose concentration was increased from 3 to 30 mmol/l, an increase in $[Ca^{2+}]_c$ was observed within 3 min in about 70 % of the cells examined (Fig. 4 A). Synchronous oscillations overlay the sustained increase in $[Ca^{2+}]_c$. During oscillations, $Ca^{2+}$ transients were synchronized among single cells in the confluent state, though there was a small time lag among these cells (Fig. 4 A, inset). The amplitudes of the $[Ca^{2+}]_c$ oscillations differed among individual cells. When $[Ca^{2+}]_c$ measured from 30 single cells was averaged, the apparent overall $[Ca^{2+}]_c$ changes induced by 30 mmol/l glucose in this pooled sample compared to those in single cells (Fig. 4 B). The mean amplitude of the $[Ca^{2+}]_c$ response to 30 mmol/l glucose was 433 ± 30 nmol/l ($n$ = 120, 30 cells in each experiment) and it was smaller than those elicited by ATP or KCl. The glucose-induced $[Ca^{2+}]_m$ increase was also much smaller (1246 ± 64 nmol/l, $n$ = 4) than those elicited by ATP or KCl and remained so for several minutes. The trace of $[Ca^{2+}]_m$ during glucose stimulation showed a markedly large oscillation amplitude superimposed on small fluctuations which were observed throughout the experiment (Fig. 4 C). After removing 30 mmol/l glucose, $[Ca^{2+}]_c$ decreased to the level which was still higher than the resting level and the oscillations disappeared. On the other hand, $[Ca^{2+}]_m$ decreased to the pre-stimulatory level after the washout. We hypothesize that the large amplitude of oscillations in $[Ca^{2+}]_m$ cannot be seen if cytosolic $Ca^{2+}$ transients are asynchronous among single cells during glucose stimulation.

In the presence of 20 mmol/l glucose, addition of 10 mmol/l KIC, a deamination product of leucine that is exclusively metabolized in mitochondria [26, 27], further increased the sustained level of $[Ca^{2+}]_c$ and the oscillation frequency in single cells (Fig. 5 A). However, $[Ca^{2+}]_c$ oscillations became obscure in averaged traces from 30 single cells because of the asynchrony of individual oscillations having an increased periodicity (Fig. 5 B). The level of $[Ca^{2+}]_m$ was raised further by KIC from the elevated level induced by glucose, while the fluctuation of

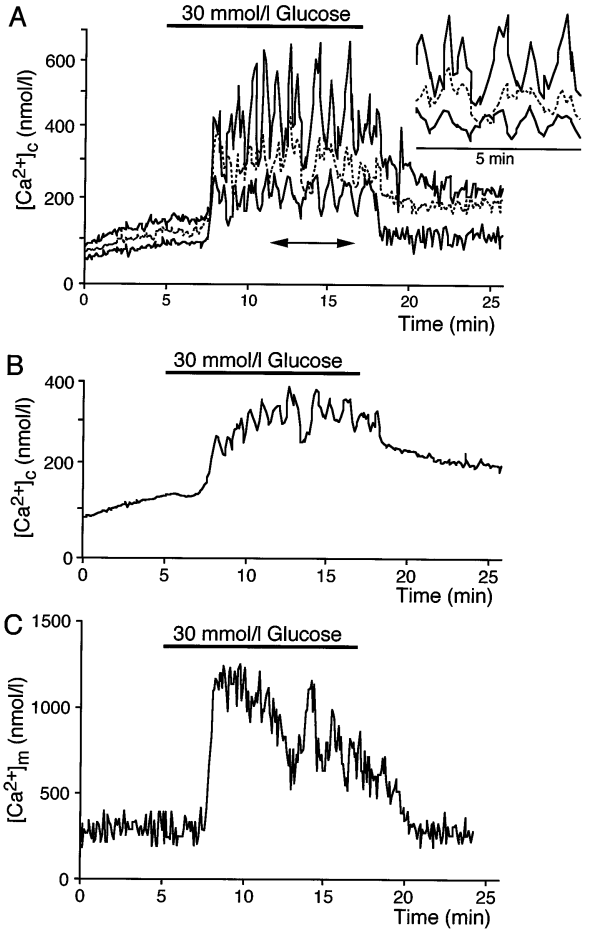


**Fig. 4 A–C.** Effects of glucose on $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$. $[Ca^{2+}]_c$ was recorded from single fura-2-loaded MIN6 cells using video imaging (**A**, data from 3 cells) and the data pooled from 30 single cells (**B**). Inset shows a time-expanded trace during the period indicated by both arrows and demonstrates synchronous $[Ca^{2+}]_c$ oscillations (**A**). $[Ca^{2+}]_m$ was measured in populations of MIN6 cells (**C**). Glucose concentration was increased from 3 to 30 mmol/l as indicated

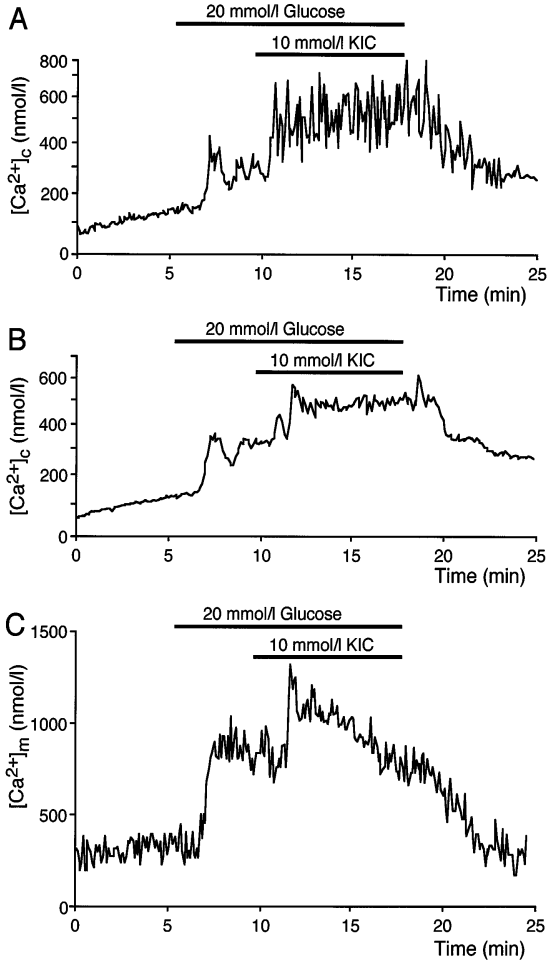


**Fig. 5.** Effects of glucose and 2-ketoisocaproic acid (KIC) on $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$. $[Ca^{2+}]_c$ was recorded from single fura-2-loaded MIN6 cells (**A**) and the data pooled from 30 single cells (**B**). $[Ca^{2+}]_m$ was measured in populations of MIN6 cells (**C**). Glucose concentration was increased from 3 to 20 mmol/l and 10 mmol/l KIC was applied as indicated

$[Ca^{2+}]_m$ during exposure to KIC was similar to that in the pre-stimulation state (Fig. 5 C).

*High KCl-induced repetitive cytosolic $Ca^{2+}$ transients caused similar transients in mitochondrial $Ca^{2+}$.* We simulated the cytosolic $[Ca^{2+}]_c$ oscillations by intermittent exposure to 22 mmol/l KCl to examine whether the mitochondrial $Ca^{2+}$ oscillates in response to cytosolic $[Ca^{2+}]_c$ oscillations. Because changes in $[Ca^{2+}]_c$ caused by pulsatile exposure to KCl were very rapid and the time lag between individual cells was negligible, the time course of $[Ca^{2+}]_c$ change in 30 cells was similar to that in single cells (Fig. 6 A, B). In this situation, $[Ca^{2+}]_m$ showed repetitive transients within the same time period as $[Ca^{2+}]_c$ (Fig. 6 C).

The increase in $[Ca^{2+}]_m$ evoked by exposure to 30 mmol/l glucose or 20 mmol/l glucose plus 10 mmol/l KIC was followed by a slow decline during either stimulation (Figs. 4 C and 5 C). During pulsatile KCl stimulations, the peak of $[Ca^{2+}]_m$ response to each pulse declined similarly in order of stimulation. The same phenomena were observed in INS-1 and HeLa cells [14, 28]. The mechanism involved in apparent desensitization of $[Ca^{2+}]_m$ is suggested to be due to the comsumption of aequorin by the first pulse in highly responsive mitochondria in which $Ca^{2+}$ influxed via the mitochondrial inner membrane can bind effectively to the aequorin. Thus, the apparent $[Ca^{2+}]_m$ signals evoked by subsequent stimulations were reduced [14].

## Discussion

We demonstrated a dynamic relationship between $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ during exposure to glucose and

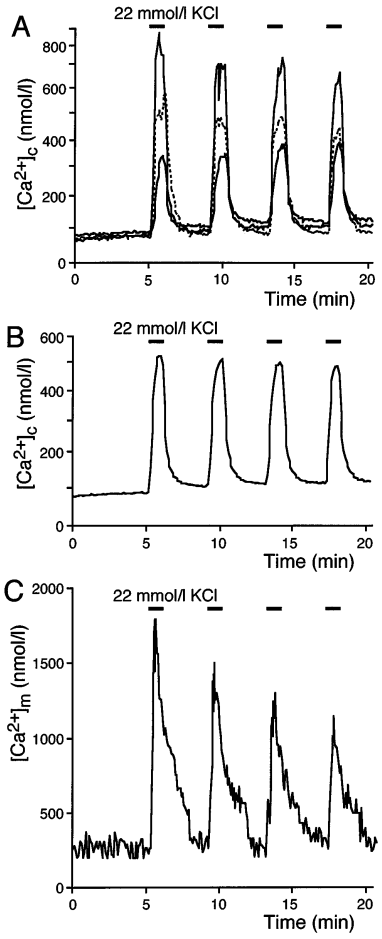


**Fig. 6.** Effects of repetitive high KCl application on $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$. $[Ca^{2+}]_c$ in single fura-2-loaded MIN6 cells was recorded (**A**, data from 3 cells) and the data pooled from 30 single cells (**B**). $[Ca^{2+}]_m$ was measured in populations of MIN6 cells (**C**). The perfusion medium contained 3 mmol/l glucose with 22 mmol/l KCl where indicated

other insulin secretagogues in the insulin-secreting cell line, MIN6. The increase in $[Ca^{2+}]_m$ depends on the $[Ca^{2+}]$, elevation induced either by an increased $Ca^{2+}$ influx via the plasma membrane or by $Ca^{2+}$ mobilization from intracellular stores. Glucose and KIC induced a persistent increase in $[Ca^{2+}]_m$, whereas ATP and KCl induced only a transient increase. Thus, the persistent and transient $[Ca^{2+}]_m$ signals appear to be related to the presence and absence of oscillatory changes in $[Ca^{2+}]_c$, respectively.

Nitrendipine attenuated the $[Ca^{2+}]_c$ increases induced by high KCl and tolbutamide but not those induced by exogenous ATP. $[Ca^{2+}]_m$ increases induced by KCl and tolbutamide were also abolished by nitrendipine, whereas the $[Ca^{2+}]_m$ increase induced by ATP remained unaffected. Extracellular ATP increases $[Ca^{2+}]_c$ by releasing $Ca^{2+}$ from intracellular $Ca^{2+}$ stores in rat islets [29]. Mechanisms vary among insulin-secreting cell lines: inositol trisphosphate mediated $Ca^{2+}$ mobilization is predominant in RINm5F cells [30, 31], while $Ca^{2+}$ influx via plasma membrane during exposure to ATP has been proposed in HIT [29] and INS-1 [23] cells. We speculate that exogenous ATP elicits $[Ca^{2+}]_m$ increase predominantly by mobilizing $Ca^{2+}$ from intracellular $Ca^{2+}$ stores in MIN6 cells, because the $[Ca^{2+}]_c$ increase was transient and unaltered by nitrendipine as observed in rat islets [29]. These results support the hypothesis that the increase of $[Ca^{2+}]_m$ depends on the increase in $[Ca^{2+}]_c$ irrespective to whether it is induced by $Ca^{2+}$ influx from the plasma membrane or $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores. Similar results have been reported for INS-1 cells [23].

$[Ca^{2+}]_m$ transients recorded with aequorin in response to KCl and extracellular ATP reached the micromolar range, whereas $[Ca^{2+}]_c$ transients measured with fura-2 rose to only a few hundred nanomolar. The discrepancy could be explained by the existence of microdomains, small domes of highly increased $[Ca^{2+}]_c$. Our $[Ca^{2+}]_c$-monitoring system using fura-2 measures spatially averaged $[Ca^{2+}]_c$ but cannot resolve changes of $[Ca^{2+}]_c$ within small areas of the cytosol. It has been shown that microdomains localized closely to the $IP_3$-gated channels of intracellular $Ca^{2+}$-stores transmit $Ca^{2+}$ into mitochondria, resulting in micromolar-range $[Ca^{2+}]_m$ in HeLa cells [32] and that higher concentrations of $Ca^{2+}$ are formed locally when $Ca^{2+}$ influx is directed toward a narrow space close to the plasma membrane in smooth muscle cells [33]. However, another possibility that the higher level of $[Ca^{2+}]_m$ than of $[Ca^{2+}]_c$ could be due to an accumulation of $Ca^{2+}$ by mitochondria cannot be excluded.

Recently, Kennedy et al. [14] demonstrated that a glucose-induced $[Ca^{2+}]_m$ increase coincides with the elevation of $[Ca^{2+}]_c$ and insulin secretion in INS-1 cells. Our observations in MIN6 cells are consistent with their results. The glucose-stimulated INS-1 cells showed asynchronous oscillation in $[Ca^{2+}]_c$ at the single cell level and a monophasic $[Ca^{2+}]_m$ increase which was the average of a group of cells [14, 23]. The present study showed, in contrast, that glucose induced a continuous $[Ca^{2+}]_m$ increase superimposed by large amplitude fluctuations exhibiting an oscillatory $[Ca^{2+}]_m$ increase. These oscillatory $[Ca^{2+}]_m$ changes were observed only at the stimulated state and apparently correlated with the $[Ca^{2+}]_c$ transients observed in a group of cells that were activated synchronously by glucose. In our experiments, MIN6 cells were fully confluent and may constitute functional cell-to-cell connections, as has been demonstrated in islet beta cells [34]. Therefore, a mass of MIN6 cells may work as a functional unit or syncytium. Synchronous $[Ca^{2+}]_c$ transients induced by glucose are observed at the single cell level in clusters of mouse beta cells [35]. The same phenomenon in $[Ca^{2+}]_c$ was observed in rodents [36–38] and human [39] islets when $[Ca^{2+}]_c$ was monitored at various regions within islets. We therefore propose that a glucose-induced

oscillatory $[Ca^{2+}]_m$ increase is produced in synchrony with cytosolic $Ca^{2+}$ oscillations. Repetitive formation of microdomains is presumably responsible for the oscillations of both $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$. This is supported by the finding that a repetitive $[Ca^{2+}]_c$ increase in response to pulsatile KCl stimulation resulted in a repetitive $[Ca^{2+}]_m$ increase. Similar oscillatory changes in $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ were observed in hepatocytes when a hormone receptor on the plasma membrane was stimulated [40].

KCl, in the absence of glucose, induces a sustained $[Ca^{2+}]_c$ increase but transient insulin secretion [41, 42]. In contrast, glucose induces $[Ca^{2+}]_c$ oscillations and a continuous oscillatory insulin release [43–46]. In the present study, KCl induced a sustained $[Ca^{2+}]_c$ increase and transient $[Ca^{2+}]_m$ increase, while glucose induced $[Ca^{2+}]_c$ oscillations and an oscillatory $[Ca^{2+}]_m$ increase. These results suggest that repetitive transients of $[Ca^{2+}]_m$ associated with $[Ca^{2+}]_c$ oscillations are necessary for continuous stimulation of mitochondrial metabolism and thereby continuous secretion of insulin.

The oscillations of $[Ca^{2+}]_c$ overlying the sustained elevation of $[Ca^{2+}]_c$, observed during glucose stimulation, appear to be more effective in maintaining $[Ca^{2+}]_m$ at high concentrations compared to the simple oscillations induced by pulsatile KCl stimulations. This may be because the sustained component of the glucose-induced $[Ca^{2+}]_c$ increase inhibits not only the mitochondrial $Ca^{2+}$ efflux but also prevents the decay of high $Ca^{2+}$ microdomains by diffusion due to a concentration difference. In the present study, both exogenous ATP and high KCl evoked transient increases of $[Ca^{2+}]_m$, whereas the time course of the $[Ca^{2+}]_m$ decline toward resting level was slower for KCl than for ATP. The $Na^+$-dependent carriers, which discharge $Ca^{2+}$ from mitochondrial matrix, are inhibited by increasing the extramitochondrial $Ca^{2+}$ concentration within the physiological range in cardiac mitochondria [47, 48]. Thus, the sustained increase in $[Ca^{2+}]_c$ induced by KCl may attenuate the movement of $Ca^{2+}$ from the mitochondrial matrix and consequently prolong the time course of the $[Ca^{2+}]_m$ decline. The existence of $Na^+$-dependent carriers has been suggested in insulinoma mitochondria [49]. It has been recently suggested that the $[Ca^{2+}]_m$ increase is required for $Ca^{2+}$-sensitive metabolic steps under physiological conditions [9, 10]. If so, the sustained component of $[Ca^{2+}]_c$ increase plays a role in keeping $[Ca^{2+}]_m$ at higher levels and therefore maintaining $Ca^{2+}$-sensitive metabolic steps in the active state during glucose stimulation.

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