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Abstract

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Desensitization of Mitochondrial Ca\(^{2+}\) and Insulin Secretion Responses in the Beta Cell*

(Received for publication, December 1, 1997, and in revised form, May 8, 1998)

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The role of mitochondria in the desensitization of insulin secretion was investigated. In rat pancreatic beta cells, both insulin secretion and mitochondrial [Ca\(^{2+}\)] increases were desensitized following two challenges with the mitochondrial substrate methyl succinate. In the beta cell line INS-1, similar results were observed when a 5-min interval separated two 5-min pulses. In contrast, ATP generation monitored in luciferase-expressing INS-1 cells was stimulated to the same extent during both exposures to methyl succinate. Succinate, like α-glycerophosphate, activates the electron transport chain at complex II. As a consequence, the mitochondrial membrane hyperpolarizes, promoting ATP synthesis and Ca\(^{2+}\) influx into the mitochondria through the uniporter. The mitochondrial desensitization was further studied in permeabilized INS-1 cells. Increasing extramitochondrial [Ca\(^{2+}\)] from 100 to 500 nM enhanced succinate oxidation 4-fold. At 500 nM Ca\(^{2+}\), 1 mM succinate caused a blunted mitochondrial [Ca\(^{2+}\)] increase upon the second, compared with the first, stimulation. These effects were mimicked by α-glycerophosphate, and there was cross-desensitization between the two compounds. Succinate hyperpolarized the mitochondrial membrane during both the first and second applications. This suggests that the uniporter itself, rather than the respiratory chain, is desensitized. These results emphasize the key role of the mitochondria not only in the stimulation of insulin secretion, but also in its desensitization.

Desensitization is a common feature of cell biology in general and of insulin secretion in particular. However, the molecular mechanism of desensitization toward nutrient stimuli is poorly understood. Nesher and Cerasi (1) first observed that successive short stimuli with glucose or arginine in the isolated perfused rat pancreas resulted in the inhibition of the insulin secretory response to the second stimulus. Insensitivity of the pancreatic beta cell to glucose was reported in pancreata taken from hyperglycemic rats (2) and is found in several diabetic animal models (3). A reduced responsiveness of the pancreatic beta cell to glucose has also been described after prolonged exposure of beta cells to hexose in vitro (4, 5) or in human subjects (6). This desensitization phenomenon is distinguished from glucose toxicity, the latter being irreversible, whereas the former implies a reversible state of cellular refractoriness due to repeated exposures to an agonist (7). Desensitization can occur at any of the multiple steps coupling glucose recognition to insulin secretion, including the exocytotic process itself, as shown in permeabilized cells exposed to repeated Ca\(^{2+}\) pulses (8).

In the pancreatic beta cell, mitochondrial metabolism plays a pivotal role in the generation of signals coupling glucose recognition to insulin secretion (9–13). The main trigger of exocytosis is an increase in cytosolic Ca\(^{2+}\) concentration (for a review, see Ref. 12). In addition, Ca\(^{2+}\) controls several other cellular functions, among them mitochondrial metabolism (14–16). An increase in mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(\text{m}\)],) following an elevation in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(\text{c}\)],) participates in the activation of the respiratory chain through stimulation of Ca\(^{2+}\)-sensitive NADH-generating dehydrogenases (15–20). NADH and FADH\(_{2}\) transfer reducing equivalents to the respiratory chain, thereby ensuring adequate ATP synthesis (15). Transfer of reducing equivalents to the electron transport chain increases the mitochondrial membrane potential (ΔΨ\(\text{m}\)), which enhances the driving force for mitochondrial Ca\(^{2+}\) uptake mediated by a low affinity uniporter (21). This ΔΨ\(\text{m}\)-dependent Ca\(^{2+}\) entry permits an amplification of [Ca\(^{2+}\)\(\text{m}\)], relative to [Ca\(^{2+}\)\(\text{c}\)], further favoring the stimulation of the aforementioned dehydrogenases (22, 23). On the other hand, the hyperpolarization of the mitochondrial membrane exerts a negative feedback by lowering the oxygen consumption and the rate of H\(^{+}\) cycling (24, 25). In glucose-stimulated beta cells, insulin secretion is initiated by the activation of mitochondrial metabolism, leading to an increase in [Ca\(^{2+}\)], (10, 26, 27). Subsequently, the rise in [Ca\(^{2+}\)\(\text{m}\)] appears to be essential for the maintenance of metabolism-secretion coupling (12, 13). The partial reduction of glucose oxidation by blockade of the [Ca\(^{2+}\)] increase (17, 28) may reflect a need for permissive [Ca\(^{2+}\)], levels in optimal glucose-stimulated insulin secretion (29).

Using cells stably expressing the calcium-sensitive photoprotein aequorin targeted to the mitochondria, we have previously shown that desensitization of insulin secretion is associated with a parallel loss of the [Ca\(^{2+}\)\(\text{m}\)] response (23). These findings and other recent studies point to a pivotal role for the mitochondria in metabolism-secretion coupling (11, 17, 20, 30–32), not only as a relay in the metabolic cascade, but also as a primary source of an as yet unidentified factor triggering insulin exocytosis (13). The existence of this putative mitochondrial factor is further suggested by studies showing impaired glucose-stimulated insulin secretion in insulinoma cells depleted of the mitochondrial genome (33, 34).

To study the involvement of the mitochondria in the desen-

* This work was supported by Swiss National Science Foundation Grants 32-32376.91 and 32-49755.96 and by a European Union Network grant (to C. B. W.) through the Swiss Federal Office for Education and Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: [Ca\(^{2+}\)\(\text{m}\)], mitochondrial Ca\(^{2+}\) concentration; [Ca\(^{2+}\)\(\text{c}\)], cytosolic Ca\(^{2+}\) concentration; ΔΨ\(\text{m}\), mitochondrial membrane potential.
situation process, we have monitored insulin secretion and three parameters that reflect mitochondrial activation: 1) $\Delta W_{\text{m}}$, using the fluorescent probe rhodamine 123, 2) [Ca$^{2+}$]$_{\text{m}}$ employing a cell line stably expressing mitochondrial aequorin, and 3) generation of ATP using a cell line stably expressing cytosolic luciferase. The tricarboxylic acid cycle intermediate succinate was used as a mitochondrial substrate. Some of these experiments were performed in cells isolated from rat islets, whereas the remainder were in cells derived from the rat insulinoma cell line INS-1 (35). To study the dissociation between the [Ca$^{2+}$]$_{\text{m}}$ signal and ATP generation, experiments were performed in Staphylococcus $\alpha$-toxin-permeabilized INS-1 cells, which permits the control of the mitochondrial environment with respect to cytosolic [Ca$^{2+}$] and [ATP] (13). The results show that [Ca$^{2+}$]$_{\text{m}}$ increases and insulin secretion are strongly desensitized by mitochondrial substrates, whereas generation of ATP and $\Delta W_{\text{m}}$ activation are not. The study provides evidence that the mitochondrial Ca$^{2+}$ uniporter is desensitized, rather than the activation of the electron transport chain.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—INS-1 cells were cultured in RPMI 1640 medium as described previously (23, 35, 36). Stable clones of INS-1 cells expressing mitochondrial aequorin (22) were established (INS-1/EK3) as detailed elsewhere (23) and cultured in the presence of 250 $\mu$g/ml G418 (Promega, Madison, WI) for continuous selection of cells expressing the plasmid with the associated neomycin resistance. Clonal INS-1 lines expressing cytosolic luciferase under the control of doxycycline-depend-ent transcriptional transactivator were established (INS-3/LUC7) (36). Following two successive stable transfections, resistant clones were cultured with 250 $\mu$g/ml G418 and 100 $\mu$g/ml hygromycin B (Calbiochem). Pancreatic islet cells were isolated by collagenase digestion from male Wistar rats weighing $\sim 200$ g (17) and cultured free floating in RPMI 1640 medium for 2–4 days.

**Transient Transfection of Primary Cells**—Rat pancreatic islet cells were isolated as described above, trypsinized, and seeded on 13-mm diameter extracellular matrix-coated coverslips (Eldan, Jerusalem, Israel) at 4 $\times$ 10$^5$ cells/ml in RPMI 1640 medium. Two days later, the cells were transfected with 10 $\mu$l of LipofectAMINE ( Gibco BRL, Basel, Switzerland) and 1 $\mu$g of vector encoding mitochondrially targeted aequorin as described previously (17). Three days later, the cells were used for the experiments. This transfection resulted in 10–15% of cells being transfected as judged by immunofluorescence of the hemaggulutinin tag incorporated at the N terminus of aequorin (22, 23).

**Permeabilization of Cells**—Attatched INS-1 cells were grown on coverslips coated with an extracellular matrix generated by confluent A431 cells, which were detached with 1% Triton X-100 (37). INS-1 cells were permeabilized after a 2–5-day culture period. Cells were first washed with an intracellular type buffer adjusted to [Ca$^{2+}$] (140 mM KCl, 5 mM NaCl, 7 mM MgSO$_4$, 20 mM HEPES, pH 7.0, 1 mM ATP, 10.2 mM EGTA, and 1.65 mM CoCl$_2$). For [Ca$^{2+}$]$_{\text{m}}$ measurements, perfusion was started with the same low Ca$^{2+}$–intracellular buffer for 2–5 min, which was then switched to the stimulatory intracellular buffer with a free Ca$^{2+}$ concentration of $\sim 500$ nM (140 mM KCl, 5 mM NaCl, 7 mM MgSO$_4$, 20 mM HEPES, pH 7.0, 10 mM ATP, 10.2 mM EGTA, and 6.67 mM CaCl$_2$).

**Measurements of Luminescence and Insulin Secretion**—Luciferase-or aequorin-expressing cells were seeded on 13-mm diameter coverslips 3–5 days prior to analysis and maintained in the same medium as described above except for the addition of G418 and hygromycin. For intact cell experiments, cells were seeded on plastic polyclinithine-treated coverslips at a density of $4 \times 10^5$ cells/ml. For permeabilized cell experiments, cells were seeded at 2 $\times$ 10$^5$ cells/ml on A431 extracellular matrix-coated coverslips as described above. Prior to luminescence measurements, buffers were maintained on an intracellular type buffer adjusted to [Ca$^{2+}$] and [glucose] + glutamine-free RPMI 1640 medium plus 10 mM HEPES for 2–5 h at 37°C. This period also served to load aequorin-expressing cells with 2.5 $\mu$m coelenterazine (Molecular Probes, Inc., Eugene, OR), the prosthetic group of aequorin (23). Luminescence was measured by placing the coverslip in a 0.5-ml thermostatted chamber at 37°C. Photon counting was done using a photon-counting board (EMI C660) prior to calibration as described previously for [Ca$^{2+}$]$_{\text{m}}$ (23). The cells were perfused constantly at a rate of 1 ml/min, and where indicated, 1-min fractions of the effluent were collected for insulin measurements. Suspensions of islet cells were preserved with the same buffer described above, except addition of 200 $\mu$m isoprenaline and 1 mM apamin (23). Intact cells were perfused with HEPES-balanced Krebs-Ringer bicarbonate buffer (135 mM NaCl, 3.6 mM KCl, 10 mM HEPES, pH 7.4, 2 mM NaHCO$_3$, 0.5 mM NaHPO$_4$, 0.5 mM MgCl$_2$, 1.5 mM CaCl$_2$, and 2.5 mM glucose) plus 10 $\mu$m bee liver luciferin (Promega) for luciferase-expressing cells. Luciferase luminescence was used for the monitoring of [ATP] in living cells as described previously (36). Permeabilized cells were measured with the intracellular buffer described above, except addition of 100 $\mu$m insulin. For insulin secretion experiments, 0.1% bovine serum albumin (Sigma) was added to buffers as carrier, and insulin was measured by radioimmunoassay using rat insulin as a standard (35).

**Mitochondrial Membrane Potential**—$\Delta W_{\text{m}}$ was measured as described (13, 39). Briefly, after a culture period in glucose-free RPMI 1640 medium, cells were loaded with 10 $\mu$M rhodamine 123 for 10 min at 37°C. For cell suspension measurements, after centrifugation, the cells were permeabilized as described above and transferred to a fluorometer cuvette, and the fluorescence excited at 490 nm was measured at 530 nm at 37°C. Following an overnight incubation in an LS-50B fluorometer (Perkin-Elmer, Buckinghamshire, United Kingdom). For measurements on attached cells, the cells grown on A431-coated glass coverslips (23) were washed with rhodamine 123 prior to the measurements (see above). Cells were then placed in a thermostatted microincubator (Medical Systems Corp., Greensville, NY) on an inverted microscope (Nikon Diaphot) with a 40× oil immersion objective. Fluorescence excitation was filtered at 485 nm, and emission was split at 505 nm and further filtered at 530 nm (Omega Optical Inc., Brattleboro, VT). The signal was recorded at 100 Hz with a photomultiplier (Nikon PI300S) and a computerized acquisition system (40). The cell layer was perfused at 1 ml/min with the 500 nM free Ca$^{2+}$–intracellular buffer described above with 0.1 $\mu$M rhodamine 123.

**Succinate Oxidation to CO$_2$ in Permeabilized INS-1 Cells**—INS-1 cells were seeded at 4 $\times$ 10$^5$ cells/ml on 35-mm diameter dishes coated with A431 extracellular matrix as described above. Cells were maintained 3 days prior to transient transfection (Fig. 1A) and cultured free floating in RPMI 1640 medium to subconfluency. Attached cells were then incubated in glucose- and glutamine-free RPMI 1640 medium plus 10 mM HEPES for 2 h at 37°C, transferred to a thermostatted glass chamber, and permeabilized according to the procedure described above. Cells were then washed with the corresponding intracellular buffer adjusted to either 100 or 500 nM free Ca$^{2+}$ and preincubated for 10 min in that buffer. Succinate oxidation was initiated by replacing the buffer with 1 ml of the respective fresh ones containing 1 mM [2-14C]succinate (NEN Life Science Products; 0.1 $\mu$Ci/chamber). After 1-h incubation at 37°C in sealed chambers, 0.5 ml of 0.1 $\mu$m HCl was added onto the cell layers to stop the reaction, and 1 ml of benzethonium hydroxide (Sigma) was injected into the bottom of the chamber to bind the CO$_2$ liberated by the oxidation (41). Following an overnight incubation at room temperature, 14CO$_2$ production was measured in benzethonium extracted with 5 ml of EtOH and counted in an LS6500 liquid scintillation counter (Beckman Instruments).

**Statistical Analysis**—Where applicable, values are expressed as the mean ± S.E., and significance of difference was calculated by Student’s t-test for unequal data. Traces without S.E. values are representative of at least three independent experiments.

**RESULTS**

**Insulin Secretion in Islets**—Rat pancreatic islets were maintained in culture for 2–4 days prior to the experiments. Stimulation of insulin secretion with 16.7 mM glucose for 10 min was repeated after a 10-min interval of perfusion at 2.8 mM glucose. This revealed that the secretory response was desensitized during the second stimulation, displaying $\sim 50\%$ reduction (Fig. 1A). The tricarboxylic acid cycle intermediate succinate, rendered cell-permeant by the ester binding of a methyl group (42), also produced a desensitization of the insulin exocytotic response with a pattern similar to that produced by glucose (Fig. 1B). Finally, KCl was used to raise [Ca$^{2+}$]$_{\text{m}}$ by membrane depolarization (29, 17). Again, the second of two exposures to 20 mM KCl revealed a blunted insulin secretory response (Fig. 1C).
Primary Pancreatic Cells—Primary rat pancreatic cells were transiently transfected with the cDNA encoding mitochondrially targeted aequorin. Monitoring of \([Ca^{2+}]_m\) in these cells showed that 5 mM methyl succinate increased \([Ca^{2+}]_m\) during the first stimulation, but not during a second one repeated 5 min later (Fig. 2A). This desensitization was also observed by raising \([Ca^{2+}]_m\) through depolarization of the plasma membrane induced by 20 mM KCl (Fig. 2B). Contrary to clones stably expressing aequorin, the low expression levels after transient transfection (13) do not permit a reliable calibration since the total photon emission was 10–20-fold less in the later case. Therefore, \([Ca^{2+}]_m\) is expressed as photons emitted per second.

ATP Generation, \([Ca^{2+}]_m\), and Insulin Secretion in INS-1 Cells—The insulin-secreting cell line INS-1 was stably transfected with mitochondrially targeted aequorin (INS-1/EK3) or with luciferase (INS-r3-LUC7), allowing the continuous measurement of \([Ca^{2+}]_m\) or [ATP], respectively, in living perfused rat islet cells. The simultaneous monitoring of \([Ca^{2+}]_m\) and insulin secretion demonstrated that both parameters exhibited an attenuated response when 5 mM methyl succinate was added to the perifusion 5 min after the first stimulation (Fig. 3, B and C, respectively). The \([Ca^{2+}]_m\) desensitization was not due to aequorin consumption or deleterious effects on the cells, as the \([Ca^{2+}]_m\) response to methyl succinate was completely restored after an interval of 30 min between the two pulses (Fig. 3D).

\([Ca^{2+}]_m\) in Permeabilized INS-1 Cells—The aequorin-expressing cells were then permeabilized with Staphylococcus α-toxin, which forms very small holes (2–3-nm diameter) in the plasma membrane (38, 8). In this preparation, the cytosolic composition and hence the mitochondrial environment can be controlled. The permeabilized cells were perfused with an intracellular type buffer containing a permissive free Ca\(^{2+}\) concentration of 500 nM and 10 mM ATP. The first addition of 1

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**Fig. 1.** Desensitization of insulin secretion in rat islets following repeated stimulation. Rat pancreatic islets were isolated and kept in culture for 2–4 days before insulin secretion experiments in a perifusion system. Stimulation with 16.7 mM Glc for 10 min was repeated after a 10-min interval (A). Using the same protocol, the effect of a 5 mM concentration of the mitochondrial substrate methyl succinate (met-Suc) is shown in B. Insulin exocytosis was also stimulated by depolarizing the cells with 20 mM KCl (C). Values are the mean ± S.E. (n = 4).

**Fig. 2.** Desensitization of \([Ca^{2+}]_m\) increases in rat islet cells. Rat islet cells were transiently transfected with the cDNA encoding mitochondrially targeted aequorin and used 3 days later. Cells were perifused at 37 °C with HEPES-balanced Krebs-Ringer bicarbonate buffer and exposed for 5 min to 5 mM methyl succinate (met-Suc) (A) or 20 mM KCl (B). Stimulation was repeated after a 5-min interval. The traces are representative of at least three independent experiments.
mM succinate induced a large transient peak in \([\text{Ca}^{2+}]_m\), but the second pulse 5 min later was ineffective (Fig. 4A). The desensitization phenomenon was also observed with \(\alpha\)-glycerophosphate, which transfers reducing equivalents from the cytosol to the same site (complex II) in the electron transport chain as succinate (Fig. 4B). Glycerophosphate has been shown to produce ATP in isolated islet mitochondria (43). More important, using 5-min intervals, succinate desensitized the effect of \(\alpha\)-glycerophosphate on \([\text{Ca}^{2+}]_m\) and vice versa (Fig. 4, C and D). This latter effect shows that the desensitization mechanism appears to be located downstream of the oxidation of FADH\(_2\). It should be noted that when \(\text{Ca}^{2+}\) was substituted with the \(\text{Ca}^{2+}\) surrogate \(\text{Sr}^{2+}\) in the intracellular type buffer, a very similar desensitization of the mitochondrial \([\text{Sr}^{2+}]_m\) increase was observed upon repeated succinate stimulation.\(^2\) As for intact cells, the desensitization was not an irreversible process due to a toxic effect or to the loss of functional aequorin since resensitization was observed after 30 min using either succinate or \(\alpha\)-glycerophosphate (Fig. 5, A and B, respectively).

To examine whether a \([\text{Ca}^{2+}]_m\) increase \textit{per se} causes desensitization to subsequent challenges, the free \(\text{Ca}^{2+}\) concentration of the buffer was varied. To this end, the extramitochondrial \([\text{Ca}^{2+}]_m\) in permeabilized cells was kept at permissive 500 nM levels or raised to 1.3 \(\mu\text{M}\). The stimulation for 5 min with 1.3 \(\mu\text{M}\) \(\text{Ca}^{2+}\) induced a first transient peak in \([\text{Ca}^{2+}]_m\) up to 1.7 \(\mu\text{M}\),

\(^2\) P. Maechler, E. D. Kennedy, and C. B. Wollheim, unpublished observations.
followed by a second phase that tended to stabilize to the level of extramitochondrial \( \text{Ca}^{2+} \). The second exposure to 1.3 mM \( \text{Ca}^{2+} \) showed a complete desensitization of the first transient increase in \( \text{[Ca}^{2+}]_m \) above the equilibration value between the two compartments (Fig. 6). Imposing the same experimental protocol except for a shortening of the exposures to 1.3 mM \( \text{Ca}^{2+} \) to 30 s instead of 5 min did not result in any desensitization of the \( \text{[Ca}^{2+}]_m \). The latter observation is in agreement with results reported for permeabilized HeLa cells expressing mitochondrial aequorin (44).

Effect of Inhibitors of the Electron Transport Chain on \( \text{[Ca}^{2+}]_m \) in Permeabilized INS-1 Cells—Succinate dehydrogenase generates FADH\(_2\), with the subsequent transfer of electrons to complex II of the electron transport chain (45). In permeabilized cells, the effect of succinate on the increase in \( \text{[Ca}^{2+}]_m \) was not affected by the presence of 100 \( \mu \text{M} \) rotenone, which blocks complex I of the respiratory chain (Fig. 7A). On the other hand, the succinate-induced \( \text{[Ca}^{2+}]_m \) increase was completely abolished by 10 \( \mu \text{M} \) antimycin A, an inhibitor of complex III (Fig. 7B). This suggests that the desensitization occurs between complex II and the uniporter, the latter mediating \( \text{Ca}^{2+} \) uptake in the mitochondria.

Effect of Free \( \text{Ca}^{2+} \) Concentration on \( \Delta \Psi_m \) and \( \text{[Ca}^{2+}]_m \) in Permeabilized INS-1 Cells—\( \text{Ca}^{2+} \) influx into the mitochondria through the uniporter is driven by the hyperpolarization of the mitochondrial membrane under conditions of permissive \( \text{[Ca}^{2+}]_m \). The hyperpolarization occurs by the transfer of reducing equivalents to the electron transport chain and the resulting extrusion of protons. We next tried to discriminate between the respiratory chain and the uniporter as the site of desensitization. For this purpose, we studied the effect of succinate on \( \text{[Ca}^{2+}]_m \) in permeabilized cells perfused with nonpermissive (resting) or permissive free \( \text{[Ca}^{2+}] \) (100 and 500 nM, respectively). Under both conditions, succinate was efficient in hyperpolarizing the mitochondrial membrane (Fig. 8, A and B). The dissipation of the proton gradient by carbonyl cyanide p-trifu-
Mitochondria play a key role in the metabolism-secretion coupling of the pancreatic beta cell (9–11, 13, 20). Evidence for a desensitization of this organelle is presented here, and the phenomenon may account for the documented desensitization of stimulated insulin secretion observed in pancreatic beta cells (1, 4, 5) and derived cell lines (23, 46). In the present study, repeated exposures of rat islets to stimulatory glucose concentrations led to attenuated insulin exocytosis. This desensitized secretory response was also observed with the mitochondrial substrate methyl succinate or with KCl-induced depolarization of the plasma membrane. In primary islet cells, desensitization of the mitochondria was indicated by the impaired rise of [Ca$^{2+}$]$_{m}$ during the second exposure to methyl succinate or high potassium. This suggests that desensitization of Ca$^{2+}$ entry into the mitochondria can be evoked by either tricarboxylic acid cycle intermediates or simply by increasing the [Ca$^{2+}$]$_{m}$. Nevertheless, to be considered a pure mitochondrial effect, the latter condition implies that the [Ca$^{2+}$]$_{m}$ increase would reach the same value during the second exposure to KCl or at least a level well above the threshold of the unipporter (400 μM) (21, 16). Although desensitization of the [Ca$^{2+}$]$_{m}$ response to KCl occurs in INS-1 cells, it still attains micromolar concentrations during the second pulse (23). The [Ca$^{2+}$]$_{m}$ reduction is less marked than that of [Ca$^{2+}$]$_{m}$, and therefore probably plays only a minor role in the mitochondrial desensitization. In intact INS-1 cells stimulated with methyl succinate, the blunted insulin secretion correlated with an inhibited increase in [Ca$^{2+}$]$_{m}$ and insulin secretion (23), but not in terms of ATP generation (see “Results”). This dichotomy between two mitochondrial parameters, [Ca$^{2+}$]$_{m}$ and ATP generation, can be explained by...
reduced Ca\(^{2+}\) uptake into the mitochondrial matrix, despite a fully activated respiratory chain. To investigate the underlying mechanism, we have used permeabilized cells to clamp extramitochondrial [Ca\(^{2+}\)] at a fixed permissive level of 500 nM. This was chosen to ascertain Ca\(^{2+}\) uptake by the uniporter (16). Under these conditions, the succinate-induced increase in [Ca\(^{2+}\)]\(_{\text{m}}\) was completely desensitized during the second stimulation. This inhibitory effect takes place downstream of complex II and is apparently not due to altered transport of succinate into the mitochondria. Indeed, the desensitizing effect of succinate could be reproduced with \(\alpha\)-glycerophosphate. This latter compound transfers reducing equivalents from the glycolytic intermediate dihydroxyacetone phosphate to the same complex II of the electron transport chain without being transported into the mitochondrial matrix (47). Thus, the desensitization evoked by both of the FADH\(_{2}\)-producing substances (succinate and \(\alpha\)-glycerophosphate) is very similar, and a common mode of action is underscored by a clear cross-desensitization. In addition, succinate-induced increases in [Ca\(^{2+}\)]\(_{\text{m}}\) were blocked by inhibiting complex III with antimycin A, but not by rotenone, which blocks complex I. We therefore conclude that the site of desensitization is located downstream of complex II either in the electron transport chain or at the uniporter through which Ca\(^{2+}\) flows into the mitochondria. The desensitization does not appear to be due to inhibition of the respiratory chain, the activation of which was not impaired. This is demonstrated by the hyperpolarization of \(\Delta\Psi_m\) irrespective of extramitochondrial Ca\(^{2+}\). Moreover, the sole hyperpolarization of \(\Delta\Psi_m\) by succinate did not attenuate the [Ca\(^{2+}\)]\(_{\text{m}}\) increase during a second exposure to the stimulus, further suggesting that the change in \(\Delta\Psi_m\) can be dissociated from the increase in [Ca\(^{2+}\)]\(_{\text{m}}\). Indeed, \(\Delta\Psi_m\) did not desensitize following two applications of succinate. The desensitization of the [Ca\(^{2+}\)]\(_{\text{m}}\) response induced by KCl in intact cells is indirect evidence for the
inhibitory effect of Ca\(^{2+}\) alone. KCl (20 mM) evokes increases in [Ca\(^{2+}\)]\(_{m}\) up to 2 \(\mu\)M (23). In permeabilized cells, direct applications of Ca\(^{2+}\) in this concentration range clearly caused desensitization of the [Ca\(^{2+}\)]\(_{m}\) response to the second pulse. This applies to the transient [Ca\(^{2+}\)]\(_{m}\) increase, but not to the equilibration of the ion between the extra- and intramitochondrial compartments, which suggests two independent pathways for mitochondrial Ca\(^{2+}\) uptake. Moreover, the desensitization requires a complete activation involving a new steady state. Indeed, very short applications (<1 min) of Ca\(^{2+}\), in the micromolar range do not lead to desensitization of [Ca\(^{2+}\)]\(_{m}\) responses during a second stimulation in permeabilized cells (44).\(^2\) Although the molecular nature of the mitochondrial Ca\(^{2+}\) uniporter has not been identified, it appears to have properties similar to those of Ca\(^{2+}\) channels of the plasma membrane (48). It may therefore be speculated that the desensitization evoked by an increase in [Ca\(^{2+}\)]\(_{m}\) could involve a mechanism similar to that described for L-type Ca\(^{2+}\) channels (49, 50). Such Ca\(^{2+}\) channel desensitization has also been reported in insulin-secreting cells (51). It is conceivable that the high frequency of the [Ca\(^{2+}\)] oscillations (two to five/min) observed in glucose-stimulated beta cells (12, 17, 52) serves to prevent desensitization of mitochondrial metabolism. It may be important to optimize the activity of the Ca\(^{2+}\)-sensitive dehydrogenases of the mitochondria to ensure the continuous production of metabolic coupling factors. We show here that succinate oxidation, reflecting tricarboxylic acid cycle activity, is stimulated by extra-mitochondrial [Ca\(^{2+}\)] in the physiological concentration range (500 nM). Such an effect was previously reported for the oxidation of pyruvate and its conversion to citrate (20).

The consensus model of metabolism-secretion coupling in the beta cell attributes a key role to ATP produced by the mitochondria (9, 10, 31). However, as clearly demonstrated by repeated stimulation with methyl succinate, ATP generation is not sufficient for the triggering of insulin secretion. Hence, in intact INS-1 cells, ATP production was preserved in the face of blunted [Ca\(^{2+}\)]\(_{m}\) and secretory responses during the second application of methyl succinate. This will result in diminished activation of the mitochondrial Ca\(^{2+}\)-sensitive dehydrogenases (15), the stimulation of which is required for full activation of the mitochondrial metabolism. An unidentified mitochondrial factor, distinct from ATP, has been proposed to participate in the triggering of insulin exocytosis (13). Its generation requires both a rise in [Ca\(^{2+}\)]\(_{m}\) and the provision of carbons to the tricarboxylic acid cycle (13). Thus, we speculate that during the desensitization of the beta cell, despite normal ATP generation, this mitochondrial factor is missing due to insufficient elevation of [Ca\(^{2+}\)]\(_{m}\). As a consequence of deficient generation of coupling factors, insulin secretion is impaired. The nature of the coupling factors of mitochondrial origin remains to be established.

Acknowledgments—We thank C. Bartley, G. Chaffard, and O. Dupont for expert technical assistance. We are also grateful to Dr. M. Palmer (University of Mainz, Mainz, Germany) for providing Staphylococcus alpha-toxin, Drs. L. Serrander and O. Nuße (University of Geneva, Geneva, Switzerland) for kind help with \(\Delta F_0\) measurements in attached cells, Dr. P. Iynedjian (University of Geneva) for INS-r3-LUC7 cells, and Dr. T. Pozzan (University of Padua, Padua, Italy) for helpful discussions.

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![Figure 9](https://example.com/figure9.png)