Requirement for Aralar and Its Ca\textsuperscript{2+}-binding Sites in Ca\textsuperscript{2+} Signal Transduction in Mitochondria from INS-1 Clonal \(\beta\)-Cells\textsuperscript{*}

Received for publication, August 29, 2008, and in revised form, November 6, 2008. Published, JBC Papers in Press, November 7, 2008, DOI 10.1074/jbc.M806729200

Patricia Mármol\textsuperscript{1,3}, Beatriz Pardo\textsuperscript{1}, Andreas Wiederkehr\textsuperscript{2,5}, Araceli del Arco\textsuperscript{1,6}, Claes B. Wollheim\textsuperscript{5,2}, and Jorgina Satrústegui\textsuperscript{1,3}

From the \textsuperscript{1}Departamento de Biología Molecular and Centro de Biología Molecular Severo Ochoa CSIC-UAM, CIBER de Enfermedades Raras (CIBERER), Universidad Autónoma, 28049 Madrid, Spain, the \textsuperscript{2}Area de Bioquímica, Centro Regional de Investigaciones Biomédicas (CRIB), Facultad de Ciencias del Medio Ambiente, Universidad de Castilla-La Mancha, 48071 Toledo, Spain, and the \textsuperscript{3}Department of Cell Physiology and Metabolism, University Medical Center, CH-1211 Geneva, Switzerland

Aralar, the mitochondrial aspartate-glutamate carrier present in \(\beta\)-cells, is a component of the malate-aspartate NADH shuttle (MAS). MAS is activated by Ca\textsuperscript{2+} in mitochondria from tissues with aralar as the only AGC isoform with an \(S_{0.5}\) of \(\approx 300\) nM. We have studied the role of aralar and its Ca\textsuperscript{2+}-binding EF-hand motifs in glucose-induced mitochondrial NAD(P)H generation by two-photon microscopy imaging in INS-1 \(\beta\)-cells lacking aralar or expressing aralar mutants blocked for Ca\textsuperscript{2+} binding. Aralar knock-down in INS-1 \(\beta\)-cell lines resulted in undetectable levels of aralar protein and complete loss of MAS activity in isolated mitochondria and in a 25% decrease in glucose-stimulated insulin secretion. MAS activity in mitochondria from INS-1 cells was activated 2–3-fold by extramitochondrial Ca\textsuperscript{2+}, whereas aralar mutants were Ca\textsuperscript{2+} insensitive. In Ca\textsuperscript{2+}-free medium, glucose-induced increases in mitochondrial NAD(P)H were small (1.3-fold) and unchanged regardless of the lack of aralar. In the presence of 1.5 mM Ca\textsuperscript{2+}, glucose induced robust increases in mitochondrial NAD(P)H (~2-fold) in cell lines with wild-type or mutant aralar. There was a \(\approx 20\%\) reduction in NAD(P)H response in cells lacking aralar, illustrating the importance of MAS in glucose action. When small Ca\textsuperscript{2+} signals that resulted in extremely small mitochondrial Ca\textsuperscript{2+} transients were induced in the presence of glucose, the rise in mitochondrial NAD(P)H was maintained in cells with wild-type aralar but was reduced by \(\approx 50\%\) in cells lacking or expressing mutant aralar. These results indicate that 1) glucose-induced activation of MAS requires Ca\textsuperscript{2+} potentiation; 2) Ca\textsuperscript{2+} activation of MAS represents a larger fraction of glucose-induced mitochondrial NAD(P)H production under conditions where suboptimal Ca\textsuperscript{2+} signals are associated with a glucose challenge (50 \textit{versus} 20%, respectively); 3) inactivation of EF-hand motifs in aralar prevents activation of MAS by small Ca\textsuperscript{2+} signals. The results suggest a possible role for aralar and MAS in priming the \(\beta\)-cell by Ca\textsuperscript{2+}-mobilizing neurotransmitter or hormones.

Glucose metabolism drives glucose-stimulated insulin secretion (GSIS)\textsuperscript{4} in pancreatic \(\beta\)-cells. The increased glucose-induced energy production results from two processes: mass action of glucose (or glucose push) and signal-dependent potentiation of metabolism (1). It is believed that mass action of glucose is governed by glucokinase as glucose sensor and increased substrate pressure is the driving force for the generation of glycolysis-derived cytosolic NADH, pyruvate formation, redox transfer to mitochondria through shuttle activity, and the glucose-induced respiratory burst (2). After the closure of ATP-dependent K\textsuperscript{+} channels (K\textsubscript{ATP} channels), Ca\textsuperscript{2+} enters in the \(\beta\)-cell (2–4) and potentiates metabolism activating mitochondrial dehydrogenases subsequent to Ca\textsuperscript{2+} inflow into the mitochondrial matrix (1). Accordingly, the initial increase of cellular NAD(P)H/NAD\textsuperscript{+}/NAD\textsuperscript{2+} (P), which is produced by mass action of glucose precedes the first cytosolic Ca\textsuperscript{2+} signal recorded (5, 6). On the other hand, glucose oxidation to CO\textsubscript{2}, which largely reflects Krebs cycle activity is markedly reduced in the absence of extracellular Ca\textsuperscript{2+}, and thus requires Ca\textsuperscript{2+} potentiation (1, 7, 8). Paradoxically, it has been reported that glucose-induced NAD(P)H production, which reflects mainly mitochondrial NAD(P)H, was not significantly reduced in the absence of extracellular Ca\textsuperscript{2+} (9). Thus, the extent to which Ca\textsuperscript{2+} signal potentiation is required for NAD(P)H production and the actual pathways involved is an open question.

Krebs cycle dehydrogenase activation may proceed through glucose mass action, as pyruvate derived from glucose is pushed into mitochondria (1) and is oxidized in the organelle forming mitochondrial NAD(P)H. However, Ca\textsuperscript{2+} activates pyruvate, isocitrate, and \(\alpha\)-ketoglutarate dehydrogenases and this is expected to potentiate mitochondrial NAD(P)H production. Redox shuttle systems, particularly the malate aspartate shuttle (MAS), are also pathways for glucose-induced NAD(P)H pro-

\textsuperscript{*} This work was supported in part by Ministerio de Educación y Ciencia Grants BFU2005-C02-01 and GEN2003-22325-C05-03/NAC, Comunidad de Madrid Grant S-GEN-0269-2006 MITOLAB-CM (to J. S.), and European Union Grant LSHM-CT-2006–518153 (to J. S. and C. B. W.). 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.

\textsuperscript{1} Recipient of a Formación de Personal Investigador fellowship from the Comunidad de Madrid.

\textsuperscript{2} Supported by Swiss National Foundation Grant 310000-116750/1.

\textsuperscript{3} To whom correspondence should be addressed. Tel.: 34-91-196-4621; Fax: 34-91-196-4420; E-mail: jsatrustegui@cbm.uam.es.

\textsuperscript{4} The abbreviations used are: GSIS, glucose-stimulated insulin secretion; AGC, aspartate-glutamate carrier; MAS, malate-aspartate NADH shuttle; Ca\textsuperscript{2+} \textsubscript{1/2}, cytosolic Ca\textsuperscript{2+}; \textsuperscript{2}Ca\textsuperscript{2+}\textsubscript{m}, mitochondrial Ca\textsuperscript{2+}; ΔΨ\textsubscript{m}, mitochondrial membrane potential; shRNA, short hairpin RNA; KD, knock-down; KRBB, Krebs-Ringer bicarbonate Hepes buffer; NADP-IDH, NADP-linked isocitrate dehydrogenase.
duction in mitochondria (10), and MAS is now known to be activated by Ca\textsuperscript{2+} (11–14). The aspartate-glutamate carrier (AGC) is one of the two mitochondrial carriers involved in MAS and catalyzes the only irreversible step in the shuttle. Aralar (also named aralar1 (11, 15)) is the AGC isomor expressed in excitable tissues, including pancreatic islets and β-cell (11, 12, 15–17). Aralar has Ca\textsuperscript{2+}-binding motifs in a long N-terminal extension of the carrier, which faces the intermembrane space and allows activation of the shuttle by cytosolic, but not mitochondrial, Ca\textsuperscript{2+} signals, in brain, skeletal muscle, and heart mitochondria (11–14).

Aralar overexpression in the β-cell increases glucose-induced rises in NAD(P)H levels and mitochondrial membrane potential, and the increase in mitochondrial activation correlates with augmentation of GSIS (17). It appears that because aralar levels in INS-1 β-cell lines and islets are lower than those found in neurons or skeletal muscle cells, increasing aralar levels augmented MAS activity and GSIS. This may result from glucose-mass action, cytosolic redox transfer keeping pace with glycolysis.

Here we have studied the impact of aralar knock-down and the importance of the intermembrane space Ca\textsuperscript{2+} binding motifs of aralar on metabolism-secretion coupling. MAS activity in mitochondria isolated from INS-1 cells was activated by extramitochondrial Ca\textsuperscript{2+} in the same range as that of brain or skeletal muscle, but not if aralar Ca\textsuperscript{2+}-binding motifs were mutated. Our results reveal that cytosolic Ca\textsuperscript{2+} binding to aralar is required for glucose-stimulated NAD(P)H transfer to mitochondria through MAS. Moreover, Ca\textsuperscript{2+} activation of shuttle activity in cells expressing wild-type but not mutated aralar accounted for most of the NAD(P)H produced in mitochondria under conditions in which glucose-induced entry of Ca\textsuperscript{2+} in mitochondria was prevented, and a small cytosolic Ca\textsuperscript{2+} signal was delivered together with glucose. The results suggest a possible role for aralar and MAS in priming the β-cell by Ca\textsuperscript{2+}-mobilizing neurotransmitter or hormones.

**EXPERIMENTAL PROCEDURES**

**Vector Construction**—To knock-down aralar, we have followed previously described methods (18) to generate aralar shRNA.pSUPER or empty pSUPER constructs from pSUPER.retro. To generate aralar shRNA.pSUPER, oligonucleotides were designed containing the aralar target sequence (shRNA A6), the mouse U6 (mU6) promoter fused to a sense strand of a 20-nucleotide sequence (GTAGCTTCTCTCCTCTCAA) followed by a short spacer (TTCAGAGA), the reverse complement of the sense strand, and five thymidines as an RNA polymerase III transcriptional stop signal. The selection of the coding sequences for shRNA was empirically determined but they started with G, contained 40–55% GC, and were analyzed by BLAST research to ensure that they did not have significant sequence homology with other genes. Plasmid PTEmU6 (Ambion) was used as a template for PCR isolation of the mU6 promoter. The PCR conditions were 96 °C denaturation for 3 min followed by 30 cycles of 96 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min; followed by one cycle of 72 °C for 10 min. The resulting PCR product was cloned into pcRII-TOPO (Invitrogen) to generate shRNA.pCRII-TOPO. To generate aralar shRNA.pSUPER, pSUPER.retro was digested with EcoRI and the shRNA cassette digested with EcoRI/EcoRI from shRNA.pCRII-TOPO was ligated into the vector. The H1 promoter from pSUPER.retro was deleted with NspV/AccI. The configuration of the construct was verified by DNA sequencing. To reconstitute the expression of wild-type aralar on cells expressing aralar shRNA.pSUPER vector, we have introduced mutations in the shRNA target sequence. To this end, mutations in the third base of the codons for Phe216, Ser217, and Tyr218 were introduced in the 1.04-kb BstEII fragment of FLAG-tagged pRESaralar1 (11, 12) by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene). The resulting sequence escapes RNA interference effects while maintaining the amino acid sequence. To generate an aralar protein inactive in Ca\textsuperscript{2+}-binding, amino acids Glu\textsuperscript{77} (position x) and Asp\textsuperscript{79} (position y) of EF-hand 1, and amino acids Asp\textsuperscript{65} (position x) and Thr\textsuperscript{67} (position y) of EF-hand 2 of the aralar sequence (11) were replaced by alanines by site-directed mutagenesis (FLAG-tagged pRESaralar1 Mut vector). FLAG-tagged pRESaralar1 and FLAG-tagged pRESaralar1 Mut vectors were confirmed by sequencing.

**Cell Culture and Transfection of INS-1 Cells**—INS-1 cells were cultured in a humidified atmosphere containing 5% CO\textsubscript{2} in a medium composed of RPMI 1640 supplemented with 10 mM Hapes, 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, and 50 µM 2-mercaptoethanol (19). The aralar shRNA.pSUPER and the empty pSUPER vector as control were transfected into the cell line INS-1 (1 µg of DNA/1 × 10\textsuperscript{6} cells). Stable cell lines expressing aralar shRNA.pSUPER (aralar KD) were selected for puromycin resistance by plating for 3 weeks in medium containing 1 µg/ml puromycin (Sigma). The FLAG-tagged pRESaralar1 and pRESaralar1 Mut vectors were transfected into the aralar KD cell line (1 µg DNA/1 × 10\textsuperscript{6} cells). Stable cell lines expressing either FLAG-tagged pRESaralar1 (Wt 24) or pRESaralar1 Mut (Mut 37) were selected for hygromycin resistance by plating for 3 weeks in media containing 200 µg/ml hygromycin (Calbiochem) and 1 µg/ml puromycin. Clonal cell lines were grown and maintained in the presence of 1 µg/ml puromycin or 1 µg/ml puromycin plus 100 µg/ml hygromycin. For all transfections Lipofectamine Plus (Invitrogen) was used. Clonal lines were isolated with cloning cylinders (Sigma) and verified by Western blotting.

**Western Blotting**—To determine the protein levels of aralar, FLAG-tagged aralar, or its Ca\textsuperscript{2+}-binding mutant, cells were homogenized in lysis buffer (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM MgCl\textsubscript{2}, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4, 1 mM iodoacetate, and 1 mM phenylmethylsulfonyl fluoride). Proteins were determined by the Bradford method. Homogenates (20 µg) were subjected to electrophoresis on a 8% polyacrylamide gel in the presence of SDS, transferred to nitrocellulose membranes (Schleicher and Schuell), and analyzed by Western blotting. Antibodies specific against aralar1 peptide-(12–343) (N-terminal extension) (15) and FLAG peptide (Sigma) were used as first antibodies (1:5,000) with goat anti-rabbit and horse anti-mouse peroxidase-conjugated IgG secondary antibodies and processed with the luminescence technique ECL (Amersham Biosciences). As control, an anti-
body against the β-subunit of F$_i$-ATPase (generous gift of Professor J. M. Cueva) was used (1:10,000). Quantitation of the bands with respect to β-subunit of F$_i$-ATPase was carried out by densitometry (GS-800 Calibrated densitometer Bio-Rad, with Bio-Rad Quantity One software).

**Isolation of Mitochondria from INS-1 Clonal β-Cells**—Cells were washed twice in homogenization buffer (250 mM sucrose, 20 mM Hepes, 2 mM EGTA, 10 mM KCl, 1.5 mM MgCl$_2$, and 0.1% bovine serum albumin, pH 7.4), harvested from the dish using a cell scraper and sedimented (350 × g for 10 min). Cells were homogenized in homogenization buffer supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM Pefabloc SC, 1 mM pepstatin A, 1 μM leupeptin, 1 μM aprotinin, and 1 μM bestatin) and nuclei, cell debris and intact cells were removed by centrifugation for 3 min at 1,090 × g. Mitochondrial fractions were then spun down (11 min, 12,100 × g), resuspended in MSK (75 mM mannitol, 25 mM sucrose, 5 mM potassium phosphate, 20 mM Tris-HCl, 0.5 mM EDTA, 100 mM KCl, and 0.1% bovine serum albumin, pH 7.4) and kept on ice until used. Proteins were measured by the Bradford method. The respiratory competence of the mitochondrial preparation was assessed by verifying succinate-dependent oxygen consumption and ADP-stimulated respiration with Clark-type electrode (21, 22).

**Reconstitution of the Malate-Aspartate NADH Shuttle Activity in Mitochondria**—MAS activity was reconstituted in mitochondria isolated from INS-1 clonal β-cells as described (13, 14, 20), except that aspartate was omitted from the assay. Mitochondrial fractions (0.4 mg of protein) were suspended in 2 ml of MSK and the shuttle was reconstituted in the presence of 4 units/ml glutamate-oxalacetate transaminase, 6 units/ml malate dehydrogenase, 66 μM NADH, 5 mM malate, 0.5 mM ADP, 200 μM ruthenium red, and appropriate CaCl$_2$ additions. MAS activity was started by the addition of 5 mM glutamate and measured as a decrease in NADH fluorescence (excitation at 340 nm, emission at 465 nm) at 37 °C under constant stirring, which was calibrated with appropriate NADH standards.

**Free Ca$^{2+}$ Calibration**—The free Ca$^{2+}$ concentrations were determined fluorimetrically with Fura-2 (below 1 μM free Ca$^{2+}$) and Calcium Green (above 1 μM free calcium) as described (13). The concentrations of Fura-2 (K$_f$ = 224 nM; excitation, 340 and 380 nm; emission, 510 nm) and Calcium Green (K$_f$ = 14 μM; excitation, 506 nm; emission, 532 nm) were 5 and 0.1 μM, respectively (Molecular Probes). The free Ca$^{2+}$ concentration was obtained by established procedures for ratiometric or non-ratiometric probes (21, 22).

**Mitochondrial Membrane Potential**—Cells were cultured in 24-well plates 48 h before the experiment. Cells were preincubated with 10 μg/ml rhodamine 123 for 20 min at 37 °C in Krebs-Ringer bicarbonate Hepes buffer (KRBB buffer; 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH$_2$PO$_4$, 0.5 mM MgSO$_4$, 1.5 mM CaCl$_2$, 2 mM NaHCO$_3$, and 10 mM Hepes, pH 7.4) and washed in the same buffer. The ΔΨ$_m$ was monitored at 37 °C in a plate reader fluorimeter (Fluostar Optima) with excitation and emission filters set at 485 and 520 nm, respectively.

**Insulin Secretion Assay**—Cells were cultured in 24-well plates coated with polyornithine 48 h before the experiment. The cells were washed and preincubated in glucose-free KRBB supplemented with 0.1% bovine serum albumin (KRBB/bovine serum albumin). Next, cells were stimulated for 30 min with 2.5 or 15 mM glucose or 30 mM KCl (in the presence of 2.5 mM glucose). Insulin secretion was determined by insulin enzyme-linked immunosorbent assay kit (SPI-BIO). Total insulin content was extracted with 10% acetic acid in ethanol (v/v). Secreted insulin was expressed as a percentage of total cellular insulin content.

**Imaging Measurements of Cytosolic Ca$^{2+}$ in INS-1 Cells**—Cells growing on coverslips coated with polylysine were loaded with 2 μM Fura-2 AM (Molecular Probes) for 40 min at 37 °C in glucose-free RPMI 1640 medium, and washed for 5–10 min in KRBB. Then coverslips were mounted in a perfusion chamber on the microscope stage as described earlier (23) and Fura-2 fluorescence was imaged ratiometrically using alternate excitation at 340 and 380 nm and a 510-nm emission filter with a Neofluar 40X/0.75 objective (13, 23) at 37 °C. Additions as indicated were made as a bolus. Single cell analysis of the changes in [Ca$^{2+}$], were expressed as the ratio of fluorescence intensity at 340 (F$_{340}$) and 380 nm (F$_{380}$) (F$_{340}$/F$_{380}$). Image acquisition and analysis were performed with the Aquacosmos 2.5 software (Hamamatsu).

**Mitochondrial Ca$^{2+}$ Measurements in INS-1 Cells**—INS-1 cells were seeded onto poly-l-ornithine-coated Thermaxon plastic coverslips (13 mm diameter, Nalgene Nunc). Attached cells were infected with an adenovirus carrying mitochondrially targeted Aequorin (24). Cells were analyzed 48 h after viral infection. The aequorin measurements were performed as described (25). Cells were loaded with coelenterazine (2.5 μM) in glucose-free RPMI 1640 for 2 h. They were then perfused with KRBB buffer (1 ml/min) at 37 °C and the Ca$^{2+}$ signal-dependent luminescence was monitored.

**Two-photon Excitation Microscopy in INS-1 Cells**—INS-1 cells were seeded on 24 × 50-mm glass coverslips at the bottom of plastic dishes that were mounted in a perfusion chamber or on glass coverslips sealed at the bottom of plastic wells (4-well LabTek chamber slide systems, NUNC). In both cases the coverslips were coated with polylysine. Cells were washed once and incubated in KRBB for 1 h before experimentation. Two-photon excitation microscopy was performed using a Nikon TE300 inverted microscope with a 60X objective and an additional digital magnification of ×2, coupled to a RTS 2000 MP (Bio-Rad) confocal/multiphoton microscopy system. An infrared multiphoton laser (Coherent Mira 900–1000 nm) provided excitation of intrinsic NADH fluorescence with a long wave excitation at 735 nm with 150-fs pulses. Images were collected with a 480/50 nm emission filter. Cells were maintained at 37 °C with a temperature-controlled microscopic stage. Additions as indicated were made as a bolus. Images (512 × 512 pixels per frame, 0.178 μm/pixel) were taken every 10 s for 400–1300 s. Image analysis was carried out with MetaMorph software (Universal Imaging). Mitochondrial intensities were determined in individual cells following procedures used in islet β-cells (26, 27) or in neurons (13). Individual cells were outlined, and intensity thresholds were set that highlighted the bright areas that corresponded to in-focus mitochondria. With these thresholds, which may underestimate the mitochondrial fluorescence but avoid contamination from the cytoplasmic compartment, the intensity of these bright areas was determined.
was calculated. Changes in mitochondrial NAD(P)H fluorescence were then quantified and normalized as $F_0/F_0$ fluorescence values.

**RESULTS**

**Knock-down of Aralar**—The aralar shRNA.pSUPER and the empty pSUPER vector as control were transfected into INS-1 clonal β-cells and stable cell lines were established. One of the different clones for aralar shRNA.pSUPER in which the levels of aralar protein was undetectable at all passage numbers tested was selected as the aralar knock-down INS-1 cell line used in this study (aralar KD) (Fig. 1A).

To obtain wild-type aralar in cells knocked-down for aralar (Wt 24), a vector carrying FLAG-tagged aralar (pRESaralar1Wt-FLAG) was transfected into aralar KD cells and stable cell lines were established. The levels of aralar protein in the Wt 24 cell line were similar to those of normal INS-1 cells as tested by Western blotting using the anti-aralar antibody (Fig. 1B).

To verify that aralar knock-down was effective MAS activity was reconstituted in mitochondria isolated from aralar KD and Wt 24 cell lines. Fig. 1C shows that MAS activity was essentially absent in mitochondrial fractions from aralar KD cells.

**Effects of Aralar KD on Mitochondrial Membrane Potential ($\Delta \Psi_m$)**—Having confirmed that knock-down of aralar was effective in abolishing MAS activity, we tested the effects of aralar deficiency on glucose-induced mitochondrial hyperpolarization. In both aralar KD and Wt 24 cells, the mitochondrial membrane was hyperpolarized by 15 mM glucose (Fig. 2A). The protonophore carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone collapsed $\Delta \Psi_m$. There were no significant differences between aralar KD and Wt 24 cells at 2.5 or 15 mM glucose (Glc), or 30 mM KCl (at 2.5 mM glucose). Data are mean ± S.E. of four independent traces. B, Wt 24 (open bars) and aralar KD (closed bars) cells were cultured in 24-well plates and assayed 48 h later. Cells were challenged for 30 min with 2.5 or 15 mM glucose (Glc), or 30 mM KCl at 2.5 mM glucose. Data are mean ± S.E. of two to three experiments performed 2–3 times. Significant differences with respect to Wt 24 cells at 15 mM glucose ($\ast$, $p < 0.05$, unpaired t test) are indicated.

**Effects of Aralar KD on Glucose-induced Insulin Secretion**—Insulin secretion in response to a stimulatory glucose concentration (15 mM) was assayed in Wt 24 and aralar KD cells over a stimulation period of 30 min and expressed as % of insulin content (Fig. 2B). At basal glucose concentration (2.5 mM glucose) 2.28 ± 0.8% insulin was released from Wt 24 cells. Stimulatory glucose (15 mM) caused an increase in insulin secretion to $9.86 \pm 0.92$% of content ($p < 0.001$). Compared with Wt 24 cells the insulin secretory response in aralar KD cells was attenuated by 25.7% ($p < 0.05$) (from 2.20 ± 0.43 at 2.5 mM glucose to 7.33 ± 0.64% of content, at 15 mM glucose, $p < 0.001$) (Fig. 2B). However, insulin release stimulated by 30 mM KCl, which rap-
**Ca**\(^{2+}\) Activation of Aralar in INS-1 Cells

**Mutation of EF-hand 1 and EF-hand 2 of Aralar—Aralar/AGC1 and citrin/AGC2 contain four pairs of EF-hands, and a single nonfunctional hypothetical EF9 in its N-terminal half (14). Deletion studies with citrin/AGC2 indicate that Ca\(^{2+}\) binding is mostly conferred by the EF1-EF2 pair, which is the only canonical EF-hand pair in aralar and citrin (14, 15).**

To study Ca\(^{2+}\) regulation of aralar, specific mutations were introduced to interfere with its ability to bind Ca\(^{2+}\) in the intermembrane space. This is illustrated in Fig. 3A. The amino acids that contribute to the octahedral Ca\(^{2+}\) co-ordination cage are labeled x, y, z, -x, -y, and -z (11, 15). To block Ca\(^{2+}\) binding to aralar, the conserved glutamate (Glu) and aspartate (Asp) at positions 27 and 29 of EF-1 (positions x and y, respectively) and the conserved aspartate (Asp) and threonine (Thr) at positions 65 and 67 of EF-hand 2 (positions x and y, respectively) of aralar were replaced by alanines (Ala) by site-directed mutagenesis to yield a FLAG-tagged aralar mutant protein (Fig. 3A).

FLAG-tagged-pRESaralar1Mut vector was transfected into aralar KD and stable cells were established (Mut 37). The levels of aralar protein in the Mut 37 cell line were similar to those of Wt 24 cells as tested by Western blotting using the anti-FLAG antibody (Fig. 3B). Verification of aralar levels in the Mut 37 cell line expressing the mutant form of the aralar protein was not possible, as the mutant protein was no longer recognized by the anti-aralar antibody (Fig. 3B).

**Ca**\(^{2+}\) Activation of the Malate-Aspartate NADH Shuttle in Isolated Mitochondria from INS-1 Cells—In tissues where aralar is the only AGC isoform, such as brain and skeletal muscle, MAS activity is activated by extramitochondrial Ca\(^{2+}\) with \(S_{0.5}\) values for Ca\(^{2+}\) activation of around 300 nm. Ca\(^{2+}\) stimulation of MAS leads to an increase in \(V_{\text{max}}\) rather than to changes in the affinity for its substrate glutamate (14).

To study Ca\(^{2+}\) activation of MAS in \(\beta\)-cell mitochondria, shuttle activity was assayed in the presence of external Ca\(^{2+}\) and ruthenium red, which blocks Ca\(^{2+}\) uptake by the Ca\(^{2+}\) uniporter in isolated mitochondria (13). Any activation caused by extramitochondrial Ca\(^{2+}\) in these conditions can be attributed to the regulation of aralar in the external face of the inner mitochondrial membrane (13, 14).

Fig. 4A shows that MAS activity in mitochondria from Wt 24 cells increased when Ca\(^{2+}\) was available in the incubation medium. The maximal activation was about 2.5-fold in response to extramitochondrial Ca\(^{2+}\) (from 4.49 ± 1.11 to 12.37 ± 1.14 nmol of NADH min\(^{-1}\) mg of protein\(^{-1}\)) in the absence of Ca\(^{2+}\) (below 10 nm) or presence of ~20 \(\mu\)M free Ca\(^{2+}\), respectively, \((p < 0.01)\) (Fig. 4B), similar to results obtained with brain mitochondria (13, 14). The activation by external Ca\(^{2+}\) was the same in the presence or absence of 200 nm ruthenium red (Fig. 4C), in agreement with the fact that the Ca\(^{2+}\) activation sites are in the intermembrane space.

To study the role of EF-hand motifs of aralar in the activation of MAS, shuttle activity was assayed in mitochondria from the Mut 37 cell line (Fig. 4D). Interestingly, Ca\(^{2+}\) stimulation of MAS activity was abolished (6.64 ± 0.77 to 5.54 ± 1.0 nmol of NADH min\(^{-1}\) mg of protein\(^{-1}\)) in the absence or in the presence of ~20 \(\mu\)M free Ca\(^{2+}\), respectively) (Fig. 4B). However, the basal activity of the shuttle in a Ca\(^{2+}\)-free medium was the same as that of the Wt 24 cell line (Fig. 4B). These results clearly show that the N-terminal EF-hand pair in aralar is responsible for Ca\(^{2+}\) activation of shuttle activity, but it is not required for the basal activity of MAS in Ca\(^{2+}\)-free conditions.

**Glucose-induced NAD(P)H Redox Transfer to Mitochondria in INS-1 Cells in a Ca\(^{2+}\)-Free Medium**—To study the role of aralar and its Ca\(^{2+}\)-binding motifs in glucose-induced redox transfer to mitochondria, we have employed conditions under which the increase in cytosolic Ca\(^{2+}\) normally produced by glucose, was avoided. This was achieved by removing extracellular Ca\(^{2+}\) (Ca\(^{2+}\)-free medium) and chelation of external Ca\(^{2+}\) with 100 \(\mu\)M EGTA at the time of glucose addition. This experimental protocol abolished the Ca\(^{2+}\) influx caused by glucose, and no increase in cytosolic ([Ca\(^{2+}\)]\(_{i}\)) or mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{m}\)) was observed in any of the INS-1 cell lines used (results not shown). Similar results have been reported both in INS-1 cell

---

**FIGURE 3. Generation of EF-hand Ca**\(^{2+}\)-binding mutant aralar and Mut 37 cells. A, alignment of the N-terminal domain of representative sequences of aralar (from human, orangutan, and mouse). Boxes indicate the position of structure loops predicted to be functional in terms of Ca\(^{2+}\) binding. The amino acids that contribute to the formation of the octahedral Ca\(^{2+}\) coordination cage are labeled x, y, z, -x, -y, and -z. Residues at the 27 and 29 positions of EF-hand 1 (Glu and Asp, respectively), and residues at the 65 and 67 positions of EF-hand 2 (Asp and Thr, respectively) were individually mutated to alanine (Ala) to disable functional Ca\(^{2+}\) binding to the respective EF-hands. B, Western blot for aralar, FLAG, and \(\beta\)-F-ATPase in Wt 24, Mut 37, empty pSUPER, and aralar KD cells. 20 \(\mu\)g of protein were used per lane and were resolved on a 8% SDS-polyacrylamide gel and immunoblotted with anti-aralar (1:5,000), anti-aralar (from human, orangutan, and mouse). Structure loops predicted to be functional in terms of Ca\(^{2+}\) binding. The active sites are in the intermembrane space.

---

**FIGURE 4. Stimulation of the shuttle by Ca**\(^{2+}\) in the absence or in the presence of glucose and Ca\(^{2+}\) chelators. A, stimulation of the shuttle by Ca\(^{2+}\) in the absence or in the presence of glucose and Ca\(^{2+}\) chelators. B, Western blot for aralar, FLAG, and \(\beta\)-F-ATPase in Wt 24, Mut 37, empty pSUPER, and aralar KD cells. 20 \(\mu\)g of protein were used per lane and were resolved on a 8% SDS-polyacrylamide gel and immunoblotted with anti-aralar (1:5,000), anti-aralar (from human, orangutan, and mouse). Structure loops predicted to be functional in terms of Ca\(^{2+}\) binding. The active sites are in the intermembrane space.

---

**TABLE 3. Generation of EF-hand Ca**\(^{2+}\)-binding mutant aralar and Mut 37 cells. A, alignment of the N-terminal domain of representative sequences of aralar (from human, orangutan, and mouse). Boxes indicate the position of structure loops predicted to be functional in terms of Ca\(^{2+}\) binding. The amino acids that contribute to the formation of the octahedral Ca\(^{2+}\) coordination cage are labeled x, y, z, -x, -y, and -z. Residues at the 27 and 29 positions of EF-hand 1 (Glu and Asp, respectively), and residues at the 65 and 67 positions of EF-hand 2 (Asp and Thr, respectively) were individually mutated to alanine (Ala) to disable functional Ca\(^{2+}\) binding to the respective EF-hands. B, Western blot for aralar, FLAG, and \(\beta\)-F-ATPase in Wt 24, Mut 37, empty pSUPER, and aralar KD cells. 20 \(\mu\)g of protein were used per lane and were resolved on a 8% SDS-polyacrylamide gel and immunoblotted with anti-aralar (1:5,000), anti-aralar (from human, orangutan, and mouse). Structure loops predicted to be functional in terms of Ca\(^{2+}\) binding. The active sites are in the intermembrane space.
Role of Cytosolic and Mitochondrial $Ca^{2+}$ in Glucose-induced NAD(P)H Redox Transfer to Mitochondria in INS-1 Cells—

To further study the regulation of glucose-stimulated MAS activity by $Ca^{2+}$ in INS-1 cells, we have employed two types of $Ca^{2+}$ signals. A very small $Ca^{2+}$ signal delivered at the time of glucose addition was obtained by adding glucose (15 mM) together with 250 $\mu$M ATP in the presence of 100 $\mu$M EGTA in $Ca^{2+}$-free KRBH. By acting on purinergic receptors, ATP evokes an inositol 1,4,5-trisphosphate-dependent release of $Ca^{2+}$ from the endoplasmic reticulum, which results in a small $Ca^{2+}$ signal in the absence of extracellular $Ca^{2+}$ (29, 30). The stimulation of glucose-induced mitochondrial NAD(P)H production obtained under these conditions was compared with that obtained under physiological conditions, in which glucose was added in a medium containing 1.5 mM $Ca^{2+}$.

Fig. 5 shows the changes in cytosolic (A–C) and mitochondrial (D–F) $Ca^{2+}$, together with the increase in mitochondrial NAD(P)H (G–I) obtained when glucose was added in the presence of $Ca^{2+}$ or in a $Ca^{2+}$-free medium. More than 85% of the Wt 24 cells analyzed (185 cells from four independent experiments) responded to 15 mM glucose in the presence of 1.5 mM extracellular $Ca^{2+}$ as shown previously in Fura-2-loaded INS-1 (29) or $\beta$-cells (31). 15 mM glucose elicited transient repetitive increases in $[Ca^{2+}]_i$ in a very large fraction of the single Wt 24 (Fig. 5B), Mut 37 (Fig. 5C), and aralar KD (Fig. 5A) clonal cell lines as previously described for INS-1 cells (25). These responses measured at the single cell level were asynchronous among the different cells (see the 3–4 different traces in Fig. 5, A–C, that correspond to individual cells). It also caused large increases in mitochondrial $Ca^{2+}$ in the whole cell population to peak levels 4–7-fold over the basal (Fig. 5, D–F), as shown earlier with mitochondrial aequorin vectors in INS-1 cell lines (25). These large increases in $[Ca^{2+}]_i$ were also essentially the same in Wt 24, Mut 37, and aralar KD cell lines. In contrast, when INS-1 cells were exposed to 15 mM glucose together with 250 $\mu$M ATP plus 100 $\mu$M EGTA in $Ca^{2+}$-free KRBH, chelation of external $Ca^{2+}$ abolished the $Ca^{2+}$ influx caused by glucose, and ATP addition resulted in a single, $Ca^{2+}$-free mitochondria. There were no significant differences in peak $[Ca^{2+}]_i$, or peak $[Ca^{2+}]_m$ obtained under these conditions when comparing aralar KD (Fig. 5, A and D), Wt 24 (Fig. 5, B and E), and Mut 37 (Fig. 5, C and F) INS-1 cells.

### Table 1

Glucose-induced peak increase in mitochondrial (Mit) NAD(P)H in the absence of extracellular $Ca^{2+}$

| Cell line    | 15 mM Glucose, 0 mM $Ca^{2+}$ | % |
|--------------|-------------------------------|---|
| Aralar KD    | 21.55 ± 2.36                  | 1.375 ± 0.02 |
| Wt 24        | 25.71 ± 5.82                  | 1.358 ± 0.19 |
| Mut 37       | 29.54 ± 7.92                  | 1.438 ± 0.03 |

A–C, comparison between $Ca^{2+}$-free and $Ca^{2+}$-containing medium; see below). The fraction of cells responding to glucose, and the peak elevation of $F/F_0$, over the basal $F/F_0$ were computed in the three cell lines. Data are mean ± S.E. of three experiments (n = 200–250 cells).

A, MAS activity in mitochondrial preparations from the Wt 24 cell line was estimated from the decrease in NAD(P)H fluorescence after glutamate (Glu) addition where indicated by the arrows. Variation of MAS activity was monitored at two free-$Ca^{2+}$ concentrations: 0 $\mu$M (i.e. below 10 nM) $Ca^{2+}$ and −20 $\mu$M $Ca^{2+}$. B, comparison between $Ca^{2+}$-activation in mitochondria from Wt 24 (open bars) and Mut 37 (closed bars) cell lines at 0 $\mu$M (i.e. below 10 nM) $Ca^{2+}$ and −20 $\mu$M $Ca^{2+}$. Data are mean ± S.E. of three experiments performed in triplicate. Significant differences with respect to basal activity (**, p < 0.01; unpaired t test) are indicated. C, comparison between $Ca^{2+}$-activation in mitochondria from the Wt 24 cell line at 0 $\mu$M (i.e. below 10 nM) $Ca^{2+}$ and −20 $\mu$M $Ca^{2+}$ in the presence (open bars) or absence (closed bars) of 200 nM ruthenium red (RR). Data are mean ± S.E. of four experiments. Significant differences with respect to basal activity (*, p < 0.05; **, p < 0.01; paired t test) are indicated. D, MAS activity in mitochondrial preparations from the Mut 37 cell line at 0 $\mu$M (i.e. below 10 nM) $Ca^{2+}$ and −20 $\mu$M $Ca^{2+}$. Traces in A and D correspond to representative experiments.

The percentage of cells showing increases in mitochondrial NAD(P)H in response to a stimulatory (15 mM) glucose concentration in these conditions was only about 25%, whereas about 70% of those cells responded to glucose in the presence of 1.5 mM extracellular $Ca^{2+}$ (see below). The fraction of cells responding in $Ca^{2+}$-free conditions was similar in Wt 24, Mut 37, and aralar KD cells (Table 1). Moreover, the glucose-induced mitochondrial NAD(P)H peak response was also much smaller in $Ca^{2+}$-free than in a $Ca^{2+}$-containing medium (about 1.35 ± 0.02-fold in $Ca^{2+}$-free versus 1.94 ± 0.11-fold in $Ca^{2+}$-containing medium; see below), and the same in all three cell lines (Table 1). The mitochondrial NAD(P)H increase is clearly dependent on cytosolic and/or mitochondrial $Ca^{2+}$. Because this increase is not reduced in aralar KD cell lines, it appears that MAS is not activated under these conditions and the increase in mitochondrial NAD(P)H in $Ca^{2+}$-free medium largely corresponds to glucose-derived pyruvate oxidation in mitochondria.
Fig. 5 shows the large increases in mitochondrial NAD(P)H elicited by glucose in single Wt 24 INS-1 cells in the presence of Ca\(^{2+}\)/H11001 and in the Ca\(^{2+}\)/H11001-free condition. Quite surprisingly, the two responses (both peak response and area under the peak) were almost identical (Fig. 5, Table 2) even though cytosolic and especially mitochondrial Ca\(^{2+}\)/H11001 signals are far smaller in the Ca\(^{2+}\)/H11001-free condition (Fig. 5, B and E). The comparison between Fig. 5, G and H, shows that knock-down of aralar results in a modest decrease in the mitochondrial NAD(P)H response elicited by glucose in the presence of Ca\(^{2+}\) but a much greater decrease in mitochondrial NAD(P)H production obtained when glucose and ATP plus EGTA were added together in a Ca\(^{2+}\)-free medium. Table 2 shows that the mitochondrial NAD(P)H response in the former condition was about 16 (peak) or 30% (area under the peak) lower than that observed in Wt 24 cells. The peak response in the Ca\(^{2+}\)-free condition was about 49 (peak) or 60% (area under the peak) lower than that in Wt 24 cells. Taken together these results clearly show that the aralar-MAS pathway accounts for about 50% of the glucose-stimulated NAD(P)H supply to mitochondria when Ca\(^{2+}\)-entry in mitochondria is strongly restricted. However, it only accounts for about ~20% of that when robust glucose-induced mitochondrial Ca\(^{2+}\) signals are generated.

Fig. 5I shows that mutations that block Ca\(^{2+}\) binding in aralar, whereas maintaining its transport activity, produce the same increase in mitochondrial NAD(P)H in response to glu-


**Ca\(^{2+}\) Activation of Aralar in INS-1 Cells**

**TABLE 2**

Glucose-induced peak increase in mitochondrial (Mit) NAD(P)H in the presence of either 1.5 mM Ca\(^{2+}\) or 250 \(\mu\)M ATP and 100 \(\mu\)M EGTA

| Cell line | 15 mM Glucose, 1.5 mM Ca\(^{2+}\) | 15 mM Glucose, 250 \(\mu\)M ATP, 100 \(\mu\)M EGTA |
|-----------|---------------------------------|-----------------------------------------------|
|           | Responding cells Mit NAD(P)H (peak (\(F_{p}\))/basal (\(F_{b}\))) Mit NAD(P)H area | Mit NAD(P)H (peak (\(F_{p}\))/basal (\(F_{b}\))) Mit NAD(P)H area |
| Aralar KD | 33.62 \pm 5.95 1.784 \pm 0.11\(^a\) 7.54 \pm 1.35\(^b\) | 1.575 \pm 0.07\(^b\) 5.33 \pm 0.55\(^b\) |
| Wt 24     | 70.12 \pm 6.12 1.941 \pm 0.07 11.344 \pm 0.89 | 2.118 \pm 0.09 14.19 \pm 1.06 |
| Mut 37    | 70.28 \pm 12.58 1.922 \pm 0.07 9.38 \pm 0.68 | 1.667 \pm 0.05\(^b\) 8.32 \pm 0.79\(^b\) |

\(\^a\) Significant differences with respect to Wt 24 cells in each condition are indicated, \(p < 0.05\), Mann-Whitney test.

\(\^b\) Significant differences with respect to Wt 24 cells in each condition are indicated, \(p < 0.001\), Mann-Whitney test.

cose in a Ca\(^{2+}\)-containing medium, as in the Wt 24 cell line (Table 2). However, when glucose was added in the Ca\(^{2+}\)-free condition the increase in mitochondrial NAD(P)H was clearly reduced, representing a \(-42\%\) decrease (peak or area under the peak) relative to Wt 24 cells (see Table 2). The response was only slightly larger than that obtained in aralar KD cells.

These results show that Ca\(^{2+}\) binding to aralar N-terminal extension is critical under the conditions in which the aralar-MAS pathway plays a major role in supplying NAD(P)H to mitochondria, *i.e.* when cytosolic Ca\(^{2+}\) signals are small and Ca\(^{2+}\) entry in mitochondria is restricted at the time of glucose addition. In fact, blocking Ca\(^{2+}\) binding to these domains results in an almost total block of the aralar-MAS pathway.

**DISCUSSION**

Aralar and mitochondrial glycerol-phosphate dehydrogenase, a mitochondrial transporter and a mitochondrial dehydrogenase, respectively, catalyze key steps in the malate-aspartate and glycerol-phosphate NADH shuttles. These shuttles are responsible for the transfer of reducing equivalents from cytosolic NADH to mitochondrial NAD(P)H and mitochondrial NADH production, regardless of MAS activity explaining the lack of effect following aralar knock-down under these conditions. In other words, it appears that the glucose push is insufficient to activate MAS. Ca\(^{2+}\) seems to be required to make it operative by increasing the \(V_{\text{max}}\) of the aralar-MAS pathway (14).

**Cytosolic Ca\(^{2+}\) Signals Are Required to Couple MAS Activity to Glucose Utilization**—The initial increase in cytosolic Ca\(^{2+}\) produced by glucose is preceded by mitochondrial activation through mass action and is followed by Ca\(^{2+}\) entry in mitochondria through the Ca\(^{2+}\) uniporter. Thus, these potentiating signals, cytosolic and mitochondrial Ca\(^{2+}\), are preceded by mitochondrial activation through mass action. We have shown that the aralar-MAS pathway is important for NAD(P)H redox transfer to \(\beta\)-cell mitochondria during glucose utilization, in particular under conditions where glucose-stimulated Ca\(^{2+}\) entry in mitochondria is prevented but ATP-induced small cytosolic Ca\(^{2+}\) transients are evoked at the time of glucose utilization. The single cytosolic Ca\(^{2+}\) transient observed under these conditions elicited a very small mitochondrial Ca\(^{2+}\) peak, much smaller than that obtained as a consequence of glucose-
induced Ca\(^{2+}\) entry into mitochondria (Fig. 5). In fact, it was surprising to find that the increase in mitochondrial NAD(P)H obtained under these conditions in Wt 24 cells is as large as that during a robust mitochondrial Ca\(^{2+}\) signal following glucose stimulation (Fig. 5H and Table 2). Our results show that the lack of aralar blocks a substantial fraction (about 50%) of the increase in mitochondrial NAD(P)H produced by glucose in the presence of these small Ca\(^{2+}\) signals, but has a much smaller effect in the presence of robust mitochondrial Ca\(^{2+}\) signals (∼20% decrease). Because the lack of aralar becomes very significant under conditions where the mitochondrial Ca\(^{2+}\) rise is drastically reduced, these results strongly suggest that the aralar-MAS pathway is responsible for conveying cytosolic rather than mitochondrial Ca\(^{2+}\) signals to mitochondria.

The role of extramitochondrial Ca\(^{2+}\) in the activity of the aralar-MAS pathway is further substantiated by our results from Mut 37 cells. Ca\(^{2+}\) activation of shuttle activity in isolated mitochondria from Mut 37 cells was completely abolished, whereas basal transport activity was similar to that of Wt 24 mitochondria. Interestingly, in intact INS-1 cells this mutant was also unable to carry out glucose-stimulated redox transfer to mitochondria in response to small Ca\(^{2+}\) signals, with a reduction in NAD(P)H transfer of about 42%.

Given the prominent role of the malate aspartate shuttle in metabolism-secretion coupling, it was surprising to find that the lack of aralar resulted only in a ∼20% decrease in glucose-induced increase in mitochondrial NAD(P)H in the presence of extracellular Ca\(^{2+}\) accompanied by a similarly small reduction in GSIS. Unlike brain mitochondria (13, 14), the maximal Ca\(^{2+}\) activation of MAS in INS-1 cell mitochondria was the same in the absence or presence of ruthenium red (Fig. 4C), indicating that matrix Ca\(^{2+}\) does not inhibit MAS activity. However, it should be noted that in the β-cell there is a pyruvate cycling pathway involving pyruvate carboxylase, the mitochondrial citrate/isocitrate carrier, cytosolic NADP-linked isocitrate dehydrogenase (NADP-IDH), cytosolic or mitochondrial malic enzyme, and possibly mitochondrial transporters of malate or malate/α-ketoglutarate (dicarboxylate carrier and oxoglutarate-malate carrier, respectively), which are involved in GSIS, especially at high glucose loads (35–40). This cycling pathway entails that some of the mitochondrial NAD(P)H producing reactions now take place in the cytosol, in fact transferring mitochondrial NADH to cytosolic NADPH and thereby reducing the impact of MAS redox shuttle on mitochondrial NAD(P)H levels. In addition, the production of α-ketoglutarate by the cytosolic NADP-IDH (36) may actually inhibit the efflux of α-ketoglutarate through the oxoglutarate-malate carrier, the second mitochondrial carrier involved in MAS, which would also limit MAS activity under conditions of potent pyruvate cycling. These reasons may explain the limited contribution of the aralar-MAS pathway to the glucose-induced increase in mitochondrial NAD(P)H in the presence of Ca\(^{2+}\) and the lack of effect of aralar Ca\(^{2+}\) binding mutants on this process.

Insulin secretion and β-cell function are sensitive to hormones and neurotransmitters that prime them to respond during the preabsorptive and absorptive phase of a meal (41). Acetylcholine or carbachol acting on M3 muscarinic G-coupled receptors, potentiates GSIS through increases in [Ca\(^{2+}\)], and increases in the efficiency of Ca\(^{2+}\)-dependent exocytosis (42). Carbachol concentrations, which induce insulin secretion even in low glucose conditions (100 μM) (43), cause rapid increases in cytosolic and mitochondrial Ca\(^{2+}\) concentrations (25, 44). These responses are further increased when carbachol is added together with glucose (25). The rapid and transient [Ca\(^{2+}\)]\(_{\text{m}}\) peak induced by carbachol arises from Ca\(^{2+}\) mobilization from the ER through the action of inositol 1,4,5-trisphosphate and preferential transfer to mitochondria (45). It strongly depends on Ca\(^{2+}\) entry into the cell, and, like ATP-induced [Ca\(^{2+}\)]\(_{\text{m}}\) signals, is reduced by blocking plasma membrane Ca\(^{2+}\) channels or in the presence of Ca\(^{2+}\)-free external medium (Ref. 25 and results not shown). Much lower concentrations of muscarinic agents (0.1–1 μM), which do not produce any stimulation of insulin release in low glucose conditions greatly potentiate glucose-stimulated insulin secretion (43) and are expected to produce much smaller cytosolic Ca\(^{2+}\) signals. It is possible that these small Ca\(^{2+}\) signals that give rise to small mitochondrial Ca\(^{2+}\) responses selectively recruit the aralar-MAS pathway to increase mitochondrial NAD(P)H. This could prime mitochondrial energization in response to glucose. We thus propose that small Ca\(^{2+}\) signals through activation of MAS could explain the potentiating effect of low levels of muscarinic and purinergic agonists on insulin release.

Acknowledgments—We thank Dr. Alberto Martínez Serrano, Dr. Helena Mira, and Dr. Ernest Arenas for help with RNA interference design; Elvira Arza, Raquel Nieto, and Dr. Alberto Álvarez Barrientos for two-photon microscopy technical support; Dr. Laura Contreras for help with reconstitution of MAS activity; and Isabel Manso, Immaculada Ocaña, and Bárbara Sesé for technical support. The CIBER de Enfermedades Raras is an initiative of the ISCIII.

REFERENCES

1. Wiederkehr, A., and Wollheim, C. B. (2008) Cell Calcium 44, 64–76
2. Matschinsky, F. M. (1996) Diabetes 45, 223–241
3. Maechler, P. (2002) Cell Mol. Life Sci. 59, 1803–1818
4. Wiederkehr, A., and Wollheim, C. B. (2006) Endocrinology 147, 2643–2649
5. Pralong, W. F., Bartley, C., and Wollheim, C. B. (1990) EMBO J. 9, 53–60
6. Pralong, W. F., Spat, A., and Wollheim, C. B. (1994) J. Biol. Chem. 269, 27310–27314
7. Hellman, B., Idahl, L. A., Lernmark, A., Sehlin, J., and Taljedal, I. B. (1974) Biochim. J. 138, 33–45
8. Sener, A., Raschaert, J., and Malaisse, W. J. (1990) Biochim. Biophys. Acta 1019, 42–50
9. Gilon, P., and Hening, J. C. (1992) J. Biol. Chem. 267, 20713–20720
10. MacDonald, M. J. (1982) Arch. Biochem. Biophys. 213, 643–649
11. del Arco, A., and Satrustegui, J. (1998) J. Biol. Chem. 273, 23237–23234
12. Palmieri, L., Pardo, B., Lasorsa, F. M., del Arco, A., Kobayashi, K., Iijima, M., Runswick, M. J., Walker, J. E., Sahedi, T., Satrustegui, J., and Palmieri, F. (2001) EMBO J. 20, 5060–5069
13. Pardo, B., Contreras, L., Serrano, A., Ramos, M., Kobayashi, K., Iijima, M., Sahedi, T., and Satrustegui, J. (2001) Biochim. Biophys. Acta 1545, 5060–5069
14. Contreras, L., Gomez-Puertas, P., Iijima, M., Kobayashi, K., Sahedi, T., and Satrustegui, J. (2007) J. Biol. Chem. 282, 7098–7106
15. Pardo, B., del Arco, A., Agudo, M., and Satrustegui, J. (2000) Biochem. J. 345, 725–732
16. del Arco, A., Morcillo, J., Martinez-Morales, J. R., Galian, C., Martos, V., Bovolenta, P., and Satrustegui, J. (2002) Eur. J. Biochem. 269, 3313–3320
17. Rubi, B., del Arco, A., Bartley, C., Satrustegui, J., and Maechler, P. (2004) J. Biol. Chem. 279, 55659–55666
Ca^{2+} Activation of Aralar in INS-1 Cells

18. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) Science 296, 550–553
19. Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P. A., and Wollheim, C. B. (1992) Endocrinology 130, 167–178
20. Jalil, M. A., Begum, L., Contreras, L., Pardo, B., Li, M. X., Ramos, M., Marmol, P., Horiuchi, M., Shimotsu, K., Nakagawa, S., Okubo, A., Sameshima, M., Isashiki, Y., Del Arco, A., Kobayashi, K., Satrustegui, J., and Saheki, T. (2005) J. Biol. Chem. 280, 31333–31339
21. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
22. Martinez, A., Vitorica, J., and Satrustegui, J. (1988) Neurosci. Lett 88, 336–342
23. Ruiz, F., Alvarez, G., Pereira, R., Hernandez, M., Villalba, M., Cruz, F., Cerdan, S., Bogoncez, E., and Satrustegui, J. (1998) Neuroreport 9, 1277–1282
24. Ishihara, H., Maechler, P., Gjinovci, A., Herrera, P. L., and Wollheim, C. B. (2003) Nat. Cell Biol. 5, 330–335
25. Kennedy, E. D., Rizzuto, R., Theler, J. M., Pralong, W. F., Bastianutto, C., Pozzan, T., and Wollheim, C. B. (1996) J. Clin. Investig. 98, 2524–2538
26. Patterson, G. H., Knobel, S. M., Arkhammar, P., Thastrup, O., and Piston, D. W. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5203–5207
27. Rocheleau, J. V., Head, W. S., Nicholson, W. E., Powers, A. C., and Piston, D. W. (2002) J. Biol. Chem. 277, 30914–30920
28. Kennedy, E. D., Maechler, P., and Wollheim, C. B. (1998) Diabetes 47, 374–380
29. Rutter, G. A., Theler, J. M., Murgia, M., Wollheim, C. B., Pozzan, T., and Rizzuto, R. (1993) J. Biol. Chem. 268, 22385–22390
30. Li, G. D., Milani, D., Dunne, M. J., Pralong, W. F., Theler, J. M., Petersen, O. H., and Wollheim, C. B. (1991) J. Biol. Chem. 266, 3449–3457
31. Theler, J. M., Mollard, P., Guerineau, N., Vacher, P., Pralong, W. F., Schlegel, W., and Wollheim, C. B. (1992) J. Biol. Chem. 267, 18110–18117
32. Eto, K., Tsukamoto, Y., Terauchi, Y., Sugiyama, T., Kishimoto, T., Taka-hashi, N., Yamauchi, N., Kubota, N., Murayama, S., Aizawa, T., Akanuma, Y., Aizawa, S., Kasai, H., Yazaki, Y., and Kadowaki, T. (1999) Science 283, 981–985
33. Heart, E., Yaney, G. C., Corkey, R. F., Schultz, V., Luc, E., Liu, L., Deeney, J. T., Shirihai, O., Tornheim, K., Smith, P. J., and Corkey, B. E. (2007) Biochem. J. 403, 197–205
34. Antinozzi, P. A., Ishihara, H., Newgard, C. B., and Wollheim, C. B. (2002) J. Biol. Chem. 277, 11746–11755
35. Schuit, F., De Vos, A., Farfari, S., Moens, K., Pipeleers, D., Brun, T., and Prentki, M. (1997) J. Biol. Chem. 272, 18572–18579
36. Ronnebaum, S. M., Ilkayeva, O., Burgess, S. C., Joseph, J. W., Lu, D., Stevens, R. D., Becker, T. C., Sherry, A. D., Newgard, C. B., and Jensen, M. V. (2006) J. Biol. Chem. 281, 30593–30602
37. Joseph, J. W., Jensen, M. V., Ilkayeva, O., Palmieri, F., Alarcon, C., Rhodes, C. J., and Newgard, C. B. (2006) J. Biol. Chem. 281, 35624–35632
38. Jensen, M. V., Joseph, J. W., Ilkayeva, O., Burgess, S., Lu, D., Ronnebaum, S. M., Odegaard, M., Becker, T. C., Sherry, A. D., and Newgard, C. B. (2006) J. Biol. Chem. 281, 22342–22351
39. Pongratz, R. L., Kibbey, R. G., Shulman, G. I., and Cline, G. W. (2007) J. Biol. Chem. 282, 200–207
40. Muoio, D. M., and Newgard, C. B. (2008) Nat. Rev. Mol. Cell. Biol. 9, 193–205
41. Ahren, B., and Holst, J. J. (2001) Diabetes 50, 1030–1038
42. Duttaroy, A., Zimliki, C. L., Gautam, D., Cui, Y., Mears, D., and Wess, J. (2004) Diabetes 53, 1714–1720
43. Garcia, M. C., Hermans, M. P., and Henquin, J. C. (1988) Biochem. J. 254, 211–218
44. Tsuiboi, T., da Silva Xavier, G., Holz, G. G., Jouaville, L. S., Thomas, A. P., and Rutter, G. A. (2003) Biochem. J. 369, 287–299
45. Rizzuto, R., and Pozzan, T. (2006) Physiol. Rev. 86, 369–408