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Recombinant Expression of the Ca^{2+}-sensitive Aspartate/Glutamate Carrier Increases Mitochondrial ATP Production in Agonist-stimulated Chinese Hamster Ovary Cells*

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The Ca^{2+}-sensitive dehydrogenases of the mitochondrial matrix are, so far, the only known effectors to allow Ca^{2+} signals to couple the activation of plasma membrane receptors to the stimulation of aerobic metabolism. In this study, we demonstrate a novel mechanism, based on Ca^{2+}-sensitive metabolite carriers of the inner membrane. We expressed in Chinese hamster ovary cells aralar1 and citrin, aspartate/glutamate exchangers that have Ca^{2+}-binding sites in their sequence, and measured mitochondrial Ca^{2+} and ATP levels as well as cytosolic Ca^{2+} concentration with targeted recombinant probes. The increase in mitochondrial ATP levels caused by cell stimulation with Ca^{2+}-mobilizing agonists was markedly larger in cells expressing aralar and citrin (but not truncated mutants lacking the Ca^{2+}-binding site) than in control cells. Conversely, the cytosolic and the mitochondrial Ca^{2+} signals were the same in control cells and cells expressing the different aralar1 and citrin variants, thus ruling out an indirect effect through the Ca^{2+}-sensitive dehydrogenases. Together, these data show that the decoding of Ca^{2+} signals in mitochondria depends on the coordinate activity of mitochondrial enzymes and carriers, which may thus represent useful pharmacological targets in this process of major pathophysiological interest.

Most intracellular ATP derives from cytosolic glycolysis and mitochondrial oxidative phosphorylation. The latter process couples the oxidation of reduced cofactors (NADH, $FADH_2$) via the respiratory chain to ATP synthesis by mitochondrial ATP synthase. The supply of reduced cofactors depends primarily on mitochondrial oxidation of substrates derived from glucose, fatty acids, and amino acids via different metabolic pathways. On the other hand, various ATP-yielding oxidative processes occurring in the cytosol, including glycolysis, cannot take place unless a proper balance between cytosolic and mitochondrial redox potential is maintained (1). Therefore, mitochondrial and cytosolic metabolisms require co-regulation at different levels. Calcium signaling seems to play a key role in this cross-talk (2–10). It has been shown that, despite the low affinity of the mitochondrial Ca^{2+} uptake systems, large increases in matrix Ca^{2+} concentration ($[Ca^{2+}]_m$) parallel the cytosolic Ca^{2+} signals after cell stimulation. In the matrix, Ca^{2+} activates three dehydrogenases of the Krebs cycle (pyruvate, isocitrate, and α-ketoglutarate dehydrogenase) (11, 12) to accommodate the higher demand for ATP production of stimulated cells. However, knowledge of the mechanism by which Ca^{2+} regulates the complex interactions between cytosolic energetic metabolism and oxidative phosphorylation is still limited (13, 14).

Cytosolic NADH is transferred into mitochondria for oxidative metabolism and ATP production through two NADH shuttles, the glycerol phosphate shuttle (15) and the malate/aspartate shuttle (16, 17). The latter requires the concerted action of two metabolite carriers in the mitochondrial inner membrane: the oxoglutarate/malate carrier (18) and the aspartate/glutamate carrier (19). Recently, we identified two closely related carrier proteins, named aralar1 and citrin, as isoforms of the mitochondrial aspartate/glutamate carrier (AGC).[1] Aralar1 (AGC1) is expressed mainly in heart, skeletal muscle, and brain, whereas citrin (AGC2) is found in many tissues but most abundantly in liver (20, 21), where abnormalities of its gene *SLC25A13* are responsible for the adult-onset type II citrullinemia (22). Both proteins have four EF-hand Ca^{2+}-binding motifs in their N-terminal domains, the characteristic features of the mitochondrial carrier family in their C-terminal domains, and both bind Ca^{2+} (20–24). The activity of AGC1 and AGC2 as aspartate/glutamate exchangers was stimulated by Ca^{2+} on the external side of the inner mitochondrial membrane, where the Ca^{2+}-binding domains of these proteins are localized (25). Based on these results, we suggested the exist-

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Dedicated to the memory of Prof. Eraldo Antonini.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AJ496568 for AGC1 and AJ496569 for AGC2.

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[1] The abbreviations used are: AGC, aspartate/glutamate carrier; AGC1, aralar1; AGC2, citrin; CHO, Chinese hamster ovary; cytAEQ, cytosolically targeted aequorin; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; mtLuc, mitochondrially targeted luciferase chimera; KRB, Krebs-Ringer buffer; mtAEQ, mitochondrially targeted aequorin; $\Delta\Psi_m$, mitochondrial membrane potential; mtGFP, mitochondrially targeted green fluorescent protein.

ence of a novel mechanism for Ca^{2+} regulation of the supply of reducing equivalents to the mitochondrial respiratory chain.

In this article, we address this issue by investigating the effect of calcium signaling on intramitochondrial ATP production in intact living cells overexpressing each of the two isoforms of AGC or of their C-terminal domains (*i.e.* the catalytic transmembrane portion, devoid of the Ca^{2+}-binding region, located in the mitochondrial intermembrane space). Using specifically targeted probes, we dynamically monitored Ca^{2+} concentration both in the cytosol and in the mitochondrial matrix as well as matrix ATP concentrations after agonist-triggered stimulation. We demonstrated that expression of either isoform of the AGC correlates with increased mitochondrial ATP production in response to the agonist stimulus. This effect required the AGC Ca^{2+}-binding regulatory domain and was more pronounced when the cells were grown in the presence of substrates that generate higher cytosolic NADH levels than glucose. We conclude that AGC plays an important role in decoding calcium signals evoked by cell stimulation into the activation of mitochondrial oxidative metabolism through the malate/aspartate NADH shuttle.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The coding sequences for AGC1 and its C-terminal domain, and for AGC2 and its C-terminal domain, were amplified by PCR from human heart and liver cDNAs, respectively. For amplification of AGC1 and AGC2, forward and reverse oligonucleotide primers corresponded to the extremities of their coding sequences (22, 23). For amplification of C-terminal domains, the forward primers corresponded to nucleotides 879–901 of the AGC1 cDNA (DDBJ/EMBL/GenBank accession number Y14494) and nucleotides 993–1012 of the AGC2 cDNA (DDBJ/EMBL/GenBank accession number AF118838). The forward and reverse primers had *Hin*dIII and *Bam*HI restriction sites, respectively. The forward primers used for amplification of AGC1 and AGC2 C-terminal domains contained an extra ATG codon immediately after the restriction site. The amplified products were *Hin*dIII/*Bam*HI-digested and cloned into the expression vector pcDNA3 (Invitrogen).

For the subcellular localization of AGC1, AGC2, and their C-terminal domains in CHO cells, the proteins were fused with EGFP at the C terminus. For this purpose, their coding sequences were amplified using modified reverse primers that did not contain the stop codon. PCR products were digested with *Hin*dIII and *Bam*HI and cloned into a modified pcDNA3 vector (26) in frame with the HA1-EGFP sequence (where HA1 is a nine-amino acid epitope derived from hemoagglutinin, and EGFP represents the humanized version of S65T GFP mutant). All clones generated by PCR amplification were verified by sequencing.

Cell Cultures and Transfection—CHO cells were grown in 75-cm^2 flasks in Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum. In parallel cultures, CHO cells were grown in the same medium supplemented with 5 mM L-malate, 5 mM L-glutamate, and 10 mM L-lactate for 10–14 days before the immunoblotting and measurement assays to increase the oxidative capacity.

For subcellular localization studies, CHO cells were grown on 24-mm round coverslips to 50–70% confluence and cotransfected according to a standard calcium-phosphate procedure (27), with 4 μg of mtEBFP/pcDNA1 (28) and 4 μg of a pcDNA3-based plasmid containing the coding sequence of either AGC1, AGC2, or their C-terminal domain fused with the EGFP sequence. Cells were processed for fluorescence microscopy 36–48 h after transfection.

For the luminescence experiments, CHO cells were allowed to attain 60–80% confluence on 13-mm coverslips and were co-transfected by the calcium-phosphate protocol with 1 μg of cytAEQ/VR1012 (29), 1 μg of mtAEQmut/VR1012 (30), or 1 μg of mtLuc/VR1012 (14) and 3 μg of a pcDNA3 plasmid harboring the sequence encoding either AGC1, AGC2, or their C-terminal domain. In control experiments, cells were co-transfected with 3 μg of mtGFP (28) and 1 μg of cytAEQ/VR1012, 1 μg of mtAEQmut/VR1012, or 1 μg mtLuc/VR1012. Aequorin and luciferase luminescence measurements were performed 36 h after transfection.

Fluorescence Microscopy—EGFP and enhanced blue fluorescent protein fluorescence was detected by an inverted Zeiss Axiovert 200 motorized microscope (Zeiss, Jena, Germany) equipped with epifluorescence. Cells were imaged with a CoolSNAP HQ CCD camera (Roper Scientific, Trenton, NJ) using the Metamorph software (Universal Imaging Corporation, Downingtown, PA).

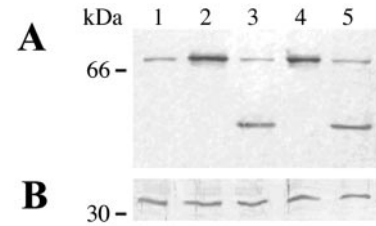

FIG. 1. **Overexpression of AGC1, AGC2, and their C-terminal domains in CHO cells.** Cells were lysed 48 h after transfection, loaded on SDS-PAGE, and transferred to nitrocellulose membrane for immunodetection with specific polyclonal anti-human AGC1 antibody (*A*) or with rabbit antiserum against bovine ADP/ATP carrier (*B*). 10 μg of protein from CHO cells transfected with pcDNA3 empty vector (*lane 1*), pcDNA3-AGC1 (*lane 2*), pcDNA3-AGC1 C-terminal domain (*lane 3*), pcDNA3-AGC2 (*lane 4*), and pcDNA3-AGC2 C-terminal domain (*lane 5*) were loaded on each lane.

Aequorin Reconstitution and Luminescence Measurements—In CHO cells transfected with cytAEQ/VR1012 or mtAEQmut/VR1012, aequorin was reconstituted by incubating the cells with 5 μM coelenterazine in Krebs-Ringer modified buffer (KRB; 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 20 mM $NaHCO_3$, 2 mM L-glutamine, 1 mM $CaCl_2$, and 20 mM HEPES, pH 7.4 at 37 °C) in a 5% CO_2 atmosphere at 37 °C for 2 h. The Ca^{2+}-dependent luminescence of aequorin was measured in cells perfused with KRB supplemented with either 5.5 mM glucose or 5 mM L-malate/5 mM L-glutamate/10 mM L-lactate. Cells were triggered by addition of ATP (100 μM) to the perfusion medium, and experiments were terminated by lysing the cells with 100 μM digitonin (Sigma-Aldrich) in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O), thus discharging the remaining aequorin pool. The light signal was collected in a purpose-built luminometer and calibrated into $[Ca^{2+}]$ values as described previously (26).

Luciferase Assays—Luciferase luminescence was measured as published (14). CHO cells co-transfected with a mitochondrially targeted luciferase chimera (mtLuc) (200,000–300,000 per coverslip) were perfused at 37 °C with KRB containing 20 μM luciferin and 1 mM $CaCl_2$ and supplemented with either 5.5 mM glucose or 5 mM L-malate/5 mM L-glutamate/10 mM L-lactate. Cellular response was evoked by the agonist ATP (100 μM) added to the perfusion medium. The light output of a coverslip of transiently transfected cells was 500–5,000 cps *versus* a background output of less than 10 cps. All compounds used in the experiments were tested for nonspecific effects on the luminescence and none were found.

Western Blot Analysis—CHO cells were lysed as described previously (31). To estimate the level of expression of AGC1, AGC2, and their C-terminal domains, proteins were separated by SDS-PAGE and immunoblotted as described previously (32). A rabbit antiserum raised against bacterially expressed human AGC1 was used.

RESULTS

Overexpression of AGC1, AGC2, and their C-terminal Domains in CHO Cells—AGC1 and AGC2 were expressed in CHO cells from the pcDNA3 vector (pcDNA3-AGC1 and pcDNA3-AGC2 plasmids). To estimate their expression level, cells were lysed 48 h after transfection, and a Western-blot analysis was performed with an antiserum raised against AGC1 (Fig. 1). The antiserum reacted equally well with AGC2, as found with *Escherichia coli*-expressed proteins (data not shown). Densitometric analysis demonstrated that CHO cells, grown in the presence of 10 mM L-lactate, 5 mM L-glutamate, and 5 mM L-malate and transfected with pcDNA3-AGC1 or pcDNA3-AGC2, showed about 3.5-fold higher expression levels for both proteins (Fig. 1*A*, *lanes 2* and *4*) compared with control cells transfected with the empty vector (Fig. 1*A*, *lane 1*). Expression levels of the C-terminal domains of AGC1 and AGC2 (Fig. 1*A*, *lanes 3* and *5*) approximately 3-fold higher were achieved under the same conditions. A somewhat lower increase in the expression of AGC1, AGC2, and their terminal domains, ~2–2.5-fold, was obtained with transfected CHO cells grown in Ham's F-12 medium (*i.e.* without respiratory sub-

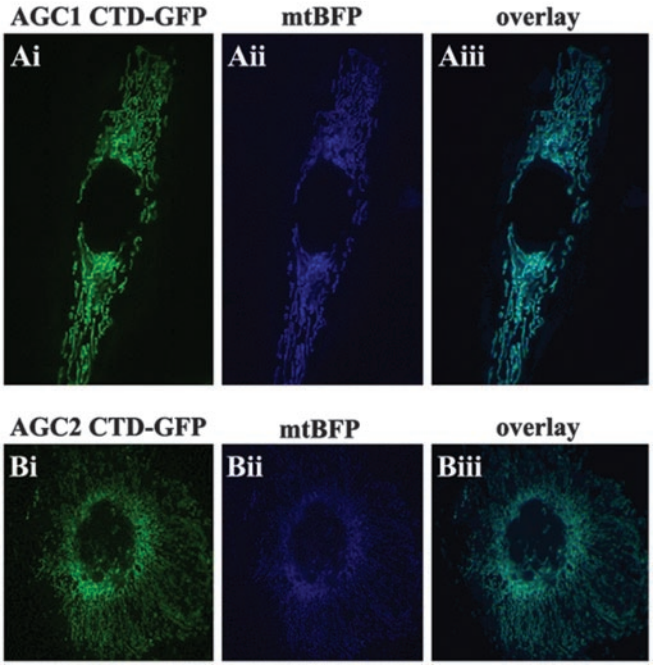

FIG. 2. **Subcellular localization of AGC1 and AGC2 C-terminal domains.** CHO cells were transiently co-transfected with pcDNA3 vectors carrying the DNA sequence coding AGC1 C-terminal domain (*A*) or AGC2 C-terminal domain (*B*) in frame with GFP DNA sequence and pcDNA1 plasmid carrying the DNA sequence of mitochondrially targeted BFP (see "Experimental Procedures"). Images were acquired by fluorescence microscopy 36–48 h after transfection and identical fields are presented. *Ai*, GFP fused AGC1 C-terminal domain fluorescence; *Bi*, GFP fused AGC2 C-terminal domain fluorescence; *Aii*, *Bii*, fluorescence of the mtBFP expressed in CHO cells; *Aiii* and *Biii*, overlaid images of mtBFP fluorescences with GFP fluorescences of AGC1 C-terminal domain and AGC2 C-terminal domain, respectively.

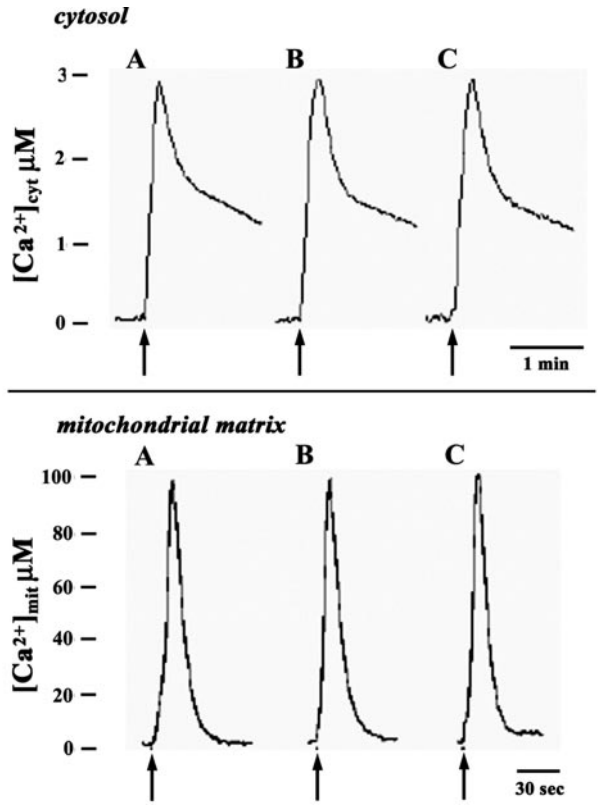

FIG. 3. **Agonist-dependent $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ changes in CHO cells overexpressing AGC1 and its C-terminal domain.** *Top* (*cytosol*), $[Ca^{2+}]_c$ measurements were carried out in CHO cells transiently co-transfected with a cytosolically targeted aequorin plasmid and mtGFP plasmid (control cells, *trace A*), or with a cytosolically targeted aequorin plasmid and pcDNA3 plasmid carrying the sequence coding AGC1 (*trace B*) or its C-terminal domain (*trace C*). *Bottom* (*mitochondrial matrix*), $[Ca^{2+}]_m$ was estimated in CHO cells transiently co-transfected with a mitochondrially targeted aequorin plasmid and mtGFP plasmid (control cells, *trace A*) or with a mitochondrially targeted aequorin plasmid and pcDNA3 plasmid carrying the sequence coding AGC1 (*trace B*) or its C-terminal domain (*trace C*). Cells were perfused with Krebs Ringer buffer supplemented with 1 mM $CaCl_2$, 5.5 mM glucose (KRB/glucose) and, where indicated (*arrow*), stimulated with 100 μM ATP. Reconstitution of the photoprotein and collection and calibration of the luminescence signal were carried out as described under "Experimental Procedures." The data are representative of 15 experiments that gave similar results.

strates). As a control, the content of the ADP/ATP carrier detected with specific antibodies (32) was essentially the same (Fig. 1*B*), indicating that the expression of AGC1 and AGC2 as well as their truncated forms does not affect the expression of other mitochondrial carriers.

Subcellular Localization of AGC1, AGC2, and Their C-terminal Domains—The intracellular localization of the ectopically expressed AGC1, AGC2, and their C-terminal domains was verified using EGFP-tagged proteins. CHO cells were transfected with pcDNA3-AGC1-EGFP and pcDNA3-AGC2-EGFP plasmids, and images were acquired 36–48 h after transfection. About 30–40% of cells were transfected, and the green fluorescence revealed a typical mitochondrial localization of the carriers (data not shown), as previously reported (20, 21). The same intracellular localization was also observed for the C-terminal domains of AGC1 and AGC2 fused with GFP, individually expressed in CHO cells (Fig. 2). The green fluorescence of the GFP-tagged proteins completely overlapped with the blue fluorescence of a mitochondrially targeted BFP (28) co-expressed in the same cells.

In vitro studies have demonstrated that the truncated forms of AGC1 and AGC2 fully retain the transport properties of the respective entire proteins (25). Our results show that removal of the Ca^{2+}-binding N-terminal domain does not prevent targeting to mitochondria of the C-terminal domains. Thereby, the carrier moieties of AGC1 and AGC2 contain in their amino acid sequences the structural information for the import into the organelle in accordance with the experimental data obtained for other mitochondrial carriers (33–35).

Cytosolic and Mitochondrial Ca^{2+} Changes in CHO Cells Overexpressing AGC1, AGC2, and Their C-terminal Domains—The presence of a long N-terminal domain containing four Ca^{2+} binding motifs is a characteristic feature of AGC1 and AGC2 that cannot be found in other hitherto-identified mitochondrial carriers. To study the physiological effects of Ca^{2+}-mediated metabolic stimulation, cells overexpressing AGC1, AGC2, or their C-terminal domain were challenged with 100 μM ATP. ATP acting on the P2Y receptor on the plasma membrane elicits IP_3-mediated mobilization of Ca^{2+} from endoplasmic reticulum (3). Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) was investigated with an aequorin chimera localized in the cytosol (29) (Fig. 3, *top*). When cells expressing AGC1 or AGC2 were perfused with Krebs-Ringer buffer supplemented with glucose and stimulated by adding ATP, $[Ca^{2+}]_c$ peak values were 2.96 ± 0.15 μM, $n = 15$ for AGC1 (Fig. 3*B*) and 2.89 ± 0.18 μM, $n = 15$ for AGC2 (data not shown). These values were essentially identical to those obtained upon stimulation of either control cells (peak value, 2.93 ± 0.13 μM, $n = 15$) (Fig. 3*A*) or cells overexpressing the C-terminal domain of AGC1 (peak value, 2.88 ± 0.16 μM, $n = 15$) (Fig. 3*C*) or AGC2 (peak value, 2.90 ± 0.09 μM, $n = 15$) (data not shown). We also monitored mitochondrial Ca^{2+} homeostasis using a mitochondrially targeted aequorin (30) (Fig. 3, *bottom*). In cells expressing AGC1 or AGC2, agonist stimulation in the presence of glucose gener-

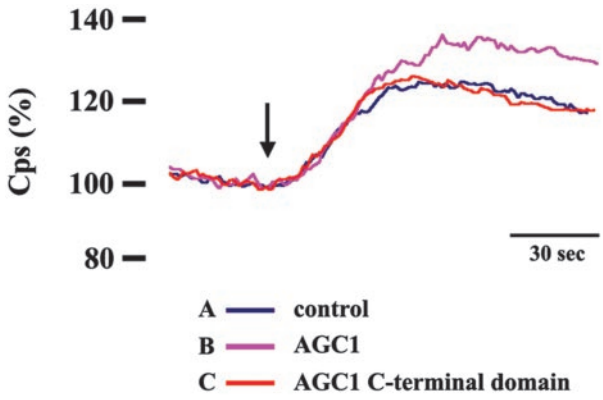

FIG. 4. **Agonist-induced mitochondrial ATP synthesis in CHO cells overexpressing AGC1 and its C-terminal domain.** ATP-dependent luciferase luminescence was measured in CHO cells transiently co-transfected with mtLuc plasmid and pcDNA3 plasmid carrying the DNA sequence of AGC1 (*trace B*) or its C-terminal domain (*trace C*). As control, cells were co-transfected with mtLuc plasmid and mt-GFP (*trace A*). Cells were perfused with KRB/glucose supplemented with 1 mM $CaCl_2$, and, where indicated (*arrow*), challenged with 100 μM ATP. Data are expressed as percentage of mtLuc light output of cells before agonist stimulation. Presented data are representative of 15 experiments that gave similar results.

ated a large transient $[Ca^{2+}]_m$ increase (peak values, 94 ± 14 μM, n = 15 for AGC1 (Fig. 3*B*) and 90 ± 18 μM, n = 15 for AGC2 (data not shown)). Virtually the same effect was obtained upon stimulation of control cells (peak value, 96 ± 16 μM, n = 15) (Fig. 3*A*). Similarly, the overexpression of the C-terminal domain of AGC1 or AGC2 did not affect the $[Ca^{2+}]_m$ increase induced by ATP stimulation (peak values, 98 ± 16 μM, n = 15 for AGC1 C-terminal domain (Fig. 3*C*) and 92 ± 15 μM, n = 15 for AGC2 C-terminal domain (data not shown)).

The effect of the presence of respiratory substrates in the growth medium on cytosolic and mitochondrial Ca^{2+} homeostasis was also studied. Control cells, grown in the presence of glutamate, malate, and lactate for 10–14 days and perfused with KRB supplemented with the same substrates, showed transient $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ increases that are virtually indistinguishable from those observed in the absence of respiratory substrates (peak values, 2.95 ± 0.19 μM, n = 8 and 98 ± 19 μM, n = 15). Therefore, as reported for other cell lines (14), growth of CHO cells in glycolytic or oxidative medium does not influence either the resting $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ or the respective agonist-dependent increases. Not surprisingly, even in the presence of glutamate, malate, and lactate, overexpression of either AGC1, AGC2, or their C-terminal domain did not cause any change in either cytosolic or mitochondrial Ca^{2+} homeostasis (data not shown). Taken together, these results indicate that the two isoforms of the mitochondrial aspartate/glutamate carrier do not affect, in either a direct or indirect way, the cytosolic Ca^{2+} content or the entry of Ca^{2+} into mitochondria.

Agonist-triggered Mitochondrial ATP Changes in CHO Cells Overexpressing AGC1 and AGC2—In CHO cells overexpressing AGC1 or AGC2, the mitochondrial ATP synthesis was dynamically monitored using mtLuc as ATP-sensitive probe (14, 36). In a first set of experiments, cells grown in Ham's F-12 medium were perfused in Krebs-Ringer buffer supplemented with glucose and stimulated by the addition of ATP. In control cells, the calcium signal induced by the agonist stimulus triggered a major synthesis of ATP in mitochondria (Fig. 4, *trace A*). This increase of mitochondrial ATP concentration (19 ± 5% of the prestimulatory value, n = 30) is very close to that reported for other cell lines (14), where it was explained with the activation of mitochondrial dehydrogenases (pyruvate, isocitrate, and α-ketoglutarate dehydrogenase) after the entry of Ca^{2+} into mitochondria via calcium uniporter. Interestingly, in CHO cells overexpressing AGC1 (Fig. 4, *trace B*) and AGC2 (data not shown), the agonist-triggered calcium signal determined a higher intramitochondrial ATP formation compared with control cells (38 ± 4% for AGC1 and 35 ± 7% for AGC2). Mitochondrial Ca^{2+} homeostasis is unvaried in CHO cells overexpressing either isoform of AGC; hence, there is no enhanced activation of mitochondrial dehydrogenases. Our results therefore indicate that the agonist-induced increase in mitochondrial ATP production observed under our conditions correlated with the expression of the two isoforms of AGC. The N-terminal domains of AGC1 and AGC2 containing the Ca^{2+}-binding sites are located on the external site of the inner mitochondrial membrane (25). Therefore, the extra production of ATP in CHO cells overexpressing AGC1 and AGC2 is caused by activation of the aspartate/glutamate carrier by Ca^{2+} from the cytosolic side.

To confirm that this is the case, the same kind of experiments were carried out with CHO cells overexpressing the C-terminal domains of either AGC1 (Fig. 4, *trace C*) or AGC2 (data not shown). In this case, the agonist-induced ATP production in mitochondria was virtually identical to that observed with control cells (22 ± 3%, n = 15 with the expressed AGC1 C-terminal domain and 17 ± 3%, n = 15 with the AGC2 C-terminal domain). The absence of the Ca^{2+}-binding regulatory domain thus abolishes the enhancement of the oxidative response, demonstrating that Ca^{2+} is the mediator of this effect. Therefore, AGC should be regarded as a bona fide mitochondrial Ca^{2+} effector that allows the Ca^{2+} signals occurring in stimulated cells to be decoded into the activation of mitochondrial metabolism.

Effect of the Presence of Malate/Aspartate NADH Shuttle Substrates on the Agonist-stimulated ATP Synthesis in CHO Cells Overexpressing AGC1 and AGC2—The aspartate/glutamate carrier plays a key role in the malate/aspartate NADH shuttle (16, 37). It is likely that Ca^{2+}-dependent activation of the AGC in turn promotes a positive regulation of the malate/aspartate NADH shuttle. Thus, the agonist-triggered $[Ca^{2+}]_c$ increases in the cytosol would allow a higher NADH/NAD ratio in the organelle, leading to increased synthesis of ATP.

To test this hypothesis, CHO cells were grown in the presence of 10 mM L-lactate, 5 mM L-glutamate, and 5 mM L-malate for 10–14 days and then perfused with Krebs-Ringer buffer supplemented with the same substrates during luciferase measurements. Under these conditions, the malate/aspartate NADH shuttle is boosted (25). In control cells challenged with the agonist ATP, the mitochondrial ATP synthesis was somewhat increased compared with the same cells grown in the absence of the oxidative substrates (30 ± 6%, n = 30) (Fig. 5, *trace A*), demonstrating the induction of an enhanced phosphorylation capacity of the cells. Under the same conditions, CHO cells overexpressing AGC1 (Fig. 5, *trace B*) and AGC2 (data not shown) exhibited a larger rise of luciferase signal after the stimulus (62 ± 8%, n = 15 for AGC1; 58 ± 6%, n = 15 for AGC2). Remarkably, mitochondrial ATP synthesis in AGC1- or AGC2-overexpressing cells was increased by 90% compared with control cells in the presence of respiratory substrates but by only 40% under glycolytic conditions. This finding can only partly be explained by the modest increase in the expression of AGC1 or AGC2 in more oxidative cells. In contrast, cells overexpressing the C-terminal domains of AGC1 (Fig. 5, *trace C*) or AGC2 (data not shown) showed agonist-induced mitochondrial ATP increases very similar to those observed in control cells (31 ± 5%, n = 15 for AGC1 C-terminal domain and 25 ± 3%, n = 15 for AGC2 C-terminal domain). Therefore, also under oxidative conditions, overexpression of truncated mitochon-

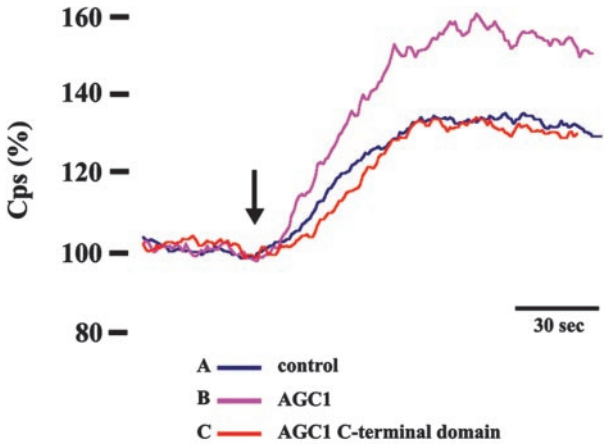

FIG. 5. **Enhanced agonist-induced mitochondrial ATP production in more oxidative CHO cells overexpressing AGC1 and its C-terminal domain.** Measurements were carried out in CHO cells grown for 10–14 days in Ham's F-12 medium supplemented with 10 mM L-lactate, 5 mM L-glutamate, and 5 mM L-malate. Thereafter, cells were transiently co-transfected with plasmids encoding mtLuc and mtGFP (control, *trace A*) or with mtLuc plasmid and pcDNA3 vector carrying the DNA sequence encoding AGC1 (*trace B*) or its C-terminal domain (*trace C*). During the assays, cells were perfused with Krebs Ringer supplemented with 1 mM $CaCl_2$ and 10 mM L-lactate, 5 mM L-glutamate, and 5 mM L-malate as mitochondrial substrates and, where indicated (*arrow*), stimulated with 100 μM ATP. Data are expressed as percentage of mtLuc light output of cells before agonist stimulation. Data shown are representative of 15 experiments that gave similar results.

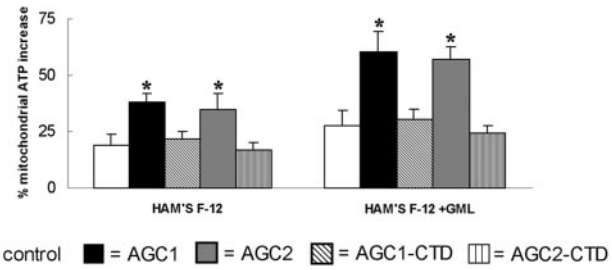

FIG. 6. **Agonist-induced mitochondrial ATP increase in CHO cells overexpressing AGC1, AGC2, and their C-terminal domains.** Measurements were carried out in CHO cells grown either in Ham's F-12 medium (*HAM'S F-12*) or for 10–14 days in the same medium supplemented with 10 mM L-lactate, 5 mM L-glutamate, and 5 mM L-malate (*HAM'S F-12 + GML*). Thereafter, cells were transiently co-transfected with plasmids encoding mtLuc and mtGFP (control) or with mtLuc plasmid and pcDNA3 vector carrying the DNA sequence encoding AGC1, AGC2, or their C-terminal domains. During the assays, cells were perfused with KRB/glucose supplemented with 1 mM $CaCl_2$ (*HAM'S F-12*) or with KRB supplemented with 1 mM $CaCl_2$ and 10 mM L-lactate, 5 mM L-glutamate, and 5 mM L-malate as mitochondrial substrates (*HAM'S F-12 + GML*) and stimulated with 100 μM ATP. Data are expressed as percentage of mtLuc light output of cells before agonist stimulation. Data shown are representative of 15 experiments that gave similar results. The significance of the difference between cells expressing AGC1 or AGC2 and controls is indicated (*, $p < 0.001$, one-way analysis of variance followed by Bonferroni t test).

drial aspartate/glutamate transporters does not affect the agonist-triggered mitochondrial ATP synthesis. The results of ATP formation in cells overexpressing AGC1, AGC2, and their C-terminal domains with or without respiratory substrates are summarized in Fig. 6. Together, these results demonstrate that the mitochondrial aspartate/glutamate transporter allows the transmission within the mitochondria of the message carried by the agonist-induced cytosolic $[Ca^{2+}]$ increases, acting as the Ca^{2+} activation site for the malate/aspartate NADH shuttle activity.

DISCUSSION

In the complex picture of Ca^{2+}-mediated modulation of mitochondrial metabolism (1, 3), the identification of AGC1 and AGC2 as Ca^{2+}-binding isoforms of the mitochondrial aspartate/glutamate carrier (AGC) (25) raises the question of their role in Ca^{2+} signaling. The AGC isoforms, which are integral proteins of the mitochondrial inner membrane, are potential sensors of $[Ca^{2+}]_c$, because they bind Ca^{2+} through their N-terminal domains, which protrude into the intermembrane space (25). AGC catalyzes a key step in the NADH malate/aspartate shuttle (16) by mediating the transport of matrix aspartate across the mitochondrial inner membrane in exchange for cytosolic glutamate. This process is electrogenic, glutamate being co-transported with one proton (19, 25), and hence far from substrate equilibrium in energized mitochondria (16). Therefore, $[Ca^{2+}]_c$ can be an important physiological effector of AGC activity given that simple changes in mass-action ratio would not affect the substrate flux through the carrier. In a previous report, we showed that the activity of AGC is stimulated by the binding of Ca^{2+} to its N-terminal domain (25). Thus, a hormone-induced increase of $[Ca^{2+}]_c$ can lead to an increased supply of reducing equivalents via the aspartate/malate shuttle through activation of AGC and consequently to an increased synthesis of mitochondrial ATP in response to cell stimulation. Indeed, in the present work, we demonstrate that in the presence of agonist-evoked Ca^{2+}-signal, ATP production in mitochondria of CHO cells overexpressing AGC1 or AGC2 is stimulated, and this increase is dependent on the presence of their Ca^{2+}-binding N-terminal domains. Our results provide a mechanistic explanation of how AGC can control the rate of the aspartate/malate shuttle, as proposed previously (16).

The stimulation of the malate/aspartate shuttle by Ca^{2+}-releasing hormones such as vasopressin, norepinephrine, and glucagon has been reported previously (38–40), but the identity of the Ca^{2+} sensor was unknown. Our results strongly suggest that α-agonists and glucagon stimulate the mitochondrial aspartate/glutamate carrier (AGC2) through an increase in $[Ca^{2+}]_c$. The same modulation of mitochondrial metabolism can take place in excitable tissues. Indeed, in heart tissue (where AGC1 is the predominant AGC isoform), Ca^{2+} has been reported to cause an increase in matrix redox potential through activation of the malate/aspartate shuttle (41). The hormone-mediated stimulation of the malate/aspartate shuttle was previously associated to the $[Ca^{2+}]_m$-mediated activation of α-ketoglutarate dehydrogenase and the consequent decrease in the level of α-ketoglutarate, which is a competitive inhibitor of the aspartate aminotransferase, an enzyme required for the operation of the malate/aspartate shuttle (42). This observation is not in contrast with the idea that AGC is a major regulator of the process. A reduced level of α-ketoglutarate because of the $[Ca^{2+}]_m$-mediated activation of α-ketoglutarate dehydrogenase would cooperate with the increased glutamate influx in mitochondria mediated by $[Ca^{2+}]_c$-stimulated AGC. Thus, Ca^{2+} would be responsible for a concerted regulation of the malate/aspartate shuttle by acting both outside (on AGC) and inside the mitochondria (on α-ketoglutarate dehydrogenase). Moreover, a transient agonist-induced increase of mitochondrial membrane potential ($\Delta\Psi_m$) has been proposed to explain the activation of malate/aspartate shuttle (38). In reality, the agonist-induced increase in $\Delta\Psi_m$ can positively regulate the malate/aspartate shuttle, given that AGC transport activity is driven by the mitochondrial proton motive force (19). However, our data indicate that an activation of α-ketoglutarate dehydrogenase or an enhanced $\Delta\Psi_m$ cannot be considered the only determinants of the activation of AGC transport activity and, hence, of the malate/aspartate shuttle. We observe no significant increase in mitochondrial ATP production upon overexpression of the C-terminal domain of either AGC isoform that

still maintains the transport features of the entire protein, including the electrogenic properties of the aspartate/glutamate exchange (25). These findings demonstrate unambiguously that Ca^{2+} binding to AGC is required for mitochondrial metabolism activation through the carrier. It should also be noted that the rise of $\Delta\Psi_m$, as observed in intact hepatocytes after Ca^{2+}-mobilizing hormone addition (13), follows a much slower kinetics than $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ responses. Therefore, the stimulatory effect of increased $\Delta\Psi_m$ takes place after the $[Ca^{2+}]_c$-mediated stimulation of AGC, sustaining the malate/aspartate shuttle activation after the $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ stimulatory effect has run out. This may explain why the activation of mitochondrial machinery after agonist stimulation continues despite the return of $[Ca^{2+}]_m$ to basal levels (13, 43). It has also been shown that the complex pattern of events after agonist stimulation evokes a transient but more prolonged increase in mitochondrial NADH; the NADH reoxidation rate in mitochondria is slow relative to the decline in $[Ca^{2+}]_m$. The more gradual reduction of mitochondrial NADH content has been interpreted as the resulting balance between a prolonged stimulation of pyruvate dehydrogenase, which is activated by a Ca^{2+}-stimulated phosphatase and inactivated by a Ca^{2+}-independent kinase (13), and an increased respiratory chain activity possibly caused by activation of complex I activity (44), leading to an increase in $\Delta\Psi_m$. Strikingly, the presence of dichloroacetate, an inhibitor of pyruvate dehydrogenase kinase, had only a limited effect on the rate of mitochondrial NADH reoxidation, suggesting that other systems contribute to the sustained increase of mitochondrial redox potential after cell stimulation (13). It is likely that AGC is one of these.

The second mitochondrial NADH shuttle system, glycerol-phosphate dehydrogenase, is also activated by extramitochondrial Ca^{2+} through an increase in the affinity for its substrate, glycerol 3-phosphate (45–47). It is reasonable to suppose a significant influence of both Ca^{2+}-regulated shuttles in the modulation of mitochondrial metabolism. This seems strengthened by the fact that individual disruption of either one of the two shuttles does not dramatically affect cellular metabolism in knock-out mice (47).[2] In liver, Sugano *et al.* (39) demonstrated that during gluconeogenesis from a reduced substrate, the α-agonist-induced Ca^{2+} mobilization mainly stimulated the malate/aspartate shuttle and only to a limited extent the α-glycerophosphate shuttle. The assessment of the relative contribution of AGC and glycerol-phosphate dehydrogenase in the Ca^{2+}-signal transduction from cytosol to mitochondria in different tissues and under different conditions deserves further investigation.

Recent studies (47, 48) indicated that (Ca^{2+}-mediated) activation of mitochondrial NADH shuttles has an important role in cell metabolism. Interestingly, in pancreatic β-cells, where AGC2 is expressed, NADH shuttles have been shown to regulate the glucose-induced insulin secretion (47). This process is promoted by glucose metabolism via the glycolytic pathway in the cytosol and via the tricarboxylic acid cycle in mitochondria. This leads to increased levels of cellular ATP, which are a major permissive factor for mobilization of insulin granules and for priming exocytosis (49, 50). To maintain high rates of glycolysis in β-cells, it is necessary that the cytosolic NADH be reoxidized; this is ensured by the mitochondrial shuttles (51). Eto and co-workers (47) showed that inhibition of both NADH shuttles leads to a severe decrease in ATP to levels that are insufficient to allow insulin secretion. Furthermore, in glucose-stimulated pancreatic β-cells, an increase in NAD(P)H cytoplasmic levels and a larger but delayed increase in mitochondrial NAD(P)H was imaged before the glucose-induced mitochondrial Ca^{2+} uptake. The separation of the glucose-stimulated cytoplasmic NAD(P)H response from the mitochondrial response (53) indicates a mechanism for the coupling of glycolytic redox equivalents into the mitochondria active before the $[Ca^{2+}]_m$ increase. It is likely that $[Ca^{2+}]_c$ spikes evoked by glucose (54) activate the malate/aspartate shuttle (as well as the glycerol-phosphate dehydrogenase shuttle), triggering the mitochondrial phase of the NAD(P)H response. This may in turn lead to an increase in the mitochondrial membrane potential, enabling Ca^{2+} influx and eventually full stimulation of the mitochondrial metabolism required for supporting the hormone secretion.

In thyroid hormone-treated cardiac mitochondria, an increased capacity of malate/aspartate and α-glycerophosphate shuttles has also been found (48). Up-regulation of the malate/aspartate shuttle was associated with increased levels of AGC protein, indicating in this carrier the regulatory element of the shuttle flux. In agreement with our results in CHO cells overexpressing AGC1 or AGC2, the increased expression of AGC1 in myocardium activates mitochondrial metabolism. Taken together, these data suggest the idea that the activity of the NADH malate/aspartate shuttle may be regulated by different mechanisms at the level of AGC.

The role of AGC in decoding calcium signals can be relevant not only for the cell energy supply in the form of increased ATP production but also for other metabolic pathways in which AGC is involved. In this regard, an example is represented by the urea cycle in the liver, where AGC catalyzes aspartate efflux from the matrix to the cytosol for incorporation into argininosuccinate (55). Thus, in the context of hepatic metabolism, AGC is likely to be an important site for the coordinated regulation by Ca^{2+} of ureogenesis, gluconeogenesis, and other Ca^{2+}-stimulated metabolic pathways, such as amino acid catabolism.

[2] T. Saheki, personal communication.

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