Ca\textsuperscript{2+}-mobilizing agonists increase mitochondrial ATP production to accelerate cytosolic Ca\textsuperscript{2+} removal: aberrations in human complex I deficiency


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Visch, Henk-Jan, Werner J. H. Koopman, Dimphy Zeegers, Sjenet E. van Emst-de Vries, Frank J. M. van Kuppeveld, Lambertus W. P. J. van den Heuvel, Jan A. M. Smeitink, and Peter H. G. M. Willems. Ca\textsuperscript{2+}-mobilizing agonists increase mitochondrial ATP production to accelerate cytosolic Ca\textsuperscript{2+} removal: aberrations in human complex I deficiency. Am J Physiol Cell Physiol 291: C308–C316, 2006. First published March 22, 2006; doi:10.1152/ajpcell.00561.2005.—Previously, we reported that both the bradykinin (Bk)-induced increase in cytosolic Ca\textsuperscript{2+} removal and the rate of cytosolic Ca\textsuperscript{2+} removal are significantly decreased in skin fibroblasts from a patient with an isolated complex I deficiency. Here we demonstrate that the mitochondrial Ca\textsuperscript{2+} indicator rhod-2 can be used to selectively buffer the Bk-induced increase in mitochondrial Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{m}) and, consequently, the Ca\textsuperscript{2+}-stimulated increase in [ATP]\textsubscript{m}, thus allowing studies of how the increase in [ATP]\textsubscript{m} and the cytosolic Ca\textsuperscript{2+} removal rate are related. Luminometry of healthy fibroblasts expressing either aequorin or luciferase in the mitochondrial matrix showed that rhod-2 dose dependently decreased the Bk-induced increase in [Ca\textsuperscript{2+}]\textsubscript{c} and [ATP]\textsubscript{m} by maximally 80 and 90%, respectively. Digital imaging microscopy of cells coloaded with the cytosolic Ca\textsuperscript{2+} indicator fura-2 revealed that, in parallel, rhod-2 maximally decreased the cytosolic Ca\textsuperscript{2+} removal rate by 20%. These findings demonstrate that increased mitochondrial ATP production is required for accelerating cytosolic Ca\textsuperscript{2+} removal during stimulation with a Ca\textsuperscript{2+}-mobilizing agonist. In contrast, complex I-deficient patient fibroblasts displayed a cytosolic Ca\textsuperscript{2+} removal rate that was already decreased by 40% compared with healthy fibroblasts. Rhod-2 did not further decrease this rate, indicating the absence of mitochondrial ATP supply to the cytosolic Ca\textsuperscript{2+} pumps. This work reveals the usefulness of rhodamine-based Ca\textsuperscript{2+} indicators in examining the role of intramitochondrial Ca\textsuperscript{2+} in mitochondrial (patho)physiology.

human skin fibroblast; OXPHOS disease; calcium ion extrusion; rhod-2; CGP-37157

Many agonists act upon receptors present on the surface of the cell to initiate a cascade of events ultimately resulting in an increase in cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{c}; see Ref. 2). This increase in [Ca\textsuperscript{2+}]\textsubscript{c} is brought about by inositol 1,4,5-trisphosphate (IP\textsubscript{3}), a water-soluble messenger that is produced at the plasma membrane from where it rapidly diffuses into the cytosol to open Ca\textsuperscript{2+} channels present in the endoplasmic reticulum (ER). The latter organelle, which forms the major intracellular Ca\textsuperscript{2+} store, actively accumulates large amounts of Ca\textsuperscript{2+} by the action of a Ca\textsuperscript{2+} pump, the sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA).

High-speed microscopy has shown that in many cells the agonist-induced increase in [Ca\textsuperscript{2+}]\textsubscript{c} starts in one region and then spreads throughout the whole cell (28, 30). Initiation of this cytosolic Ca\textsuperscript{2+} wave occurs at a site where IP\textsubscript{3} channel density is high and/or IP\textsubscript{3} channels are most sensitive to activation by IP\textsubscript{3}. Subsequently, the cytosolic Ca\textsuperscript{2+} signal propagates by a mechanism referred to as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release. Whether or not a global Ca\textsuperscript{2+} wave arises depends on the Ca\textsuperscript{2+} concentration that is reached in the initiation region. This, in turn, depends on the number of Ca\textsuperscript{2+} channels that is opened by IP\textsubscript{3}, which, under the given conditions, depends on the IP\textsubscript{3} concentration and thus on the agonist concentration, the local SERCA activity, and the local concentration of cytosolic Ca\textsuperscript{2+} buffers.

Several studies have shown that the agonist-induced cytosolic Ca\textsuperscript{2+} signal can be confined to the initiation region by strategically positioned mitochondria (28, 30). This function of mitochondria critically depends on the potential difference across the inner mitochondrial membrane as was demonstrated by the action of mitochondrial uncouplers and inhibitors of the electron transport chain. Mitochondrial Ca\textsuperscript{2+} uptake not only controls the local [Ca\textsuperscript{2+}]\textsubscript{c} and, in doing so, the spreading of the cytosolic Ca\textsuperscript{2+} signal, but also regulates the activity of several enzymes involved in mitochondrial ATP production (5, 8, 11, 12, 25, 26, 32, 33). In addition, Ca\textsuperscript{2+} has been postulated to regulate mitochondrial ATP production through a mechanism independent of mitochondrial Ca\textsuperscript{2+} uptake, involving the activation of the aspartate-malate NADH shuttle by Ca\textsuperscript{2+} on the external side of the inner mitochondrial membrane (15, 19). At present, however, the relative importance of this shuttle in agonist-induced mitochondrial ATP production is unclear.

To enhance our understanding of the pathophysiology of disorders of the human oxidative phosphorylation (OXPHOS) system, we study genetically characterized patient skin fibroblasts (13, 14, 27, 32, 33). In doing so, we recently showed that agonist-induced mitochondrial Ca\textsuperscript{2+} accumulation and ensuing ATP production are significantly reduced in fibroblasts from patients with a mutation in nuclear encoded subunits of complex I (NADH-ubiquinone oxidoreductase, EC 1.6.5.3) of the OXPHOS system (OMIM 252010; see Refs. 32 and 33). In addition, we found that the rate of Ca\textsuperscript{2+} removal from the

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cytosolic compartment was significantly decreased in these patient fibroblasts. Because the latter process involves the action of ATP-dependent Ca\(^{2+}\) transporters, of which the plasma membrane Ca\(^{2+}\)-ATPase and the SERCA are the most prominent ones, we concluded that the observed reduction in cytosolic Ca\(^{2+}\) removal rate was the result of impaired fueling of these transporters. This conclusion was supported by the finding that normalization of the agonist-induced increase in mitochondrial ATP concentration ([ATP\(_m\)]) by the inhibitor of mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange, CGP-37157, restored the rate of cytosolic Ca\(^{2+}\) removal.

To more firmly establish the relationship between Ca\(^{2+}\)-stimulated mitochondrial ATP production and the rate of cytosolic Ca\(^{2+}\) removal, we sought a means to prevent the agonist-induced increase in mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_m\)]), thereby abolishing the ensuing increase in mitochondrial ATP production. The data presented show that the mitochondrial Ca\(^{2+}\) indicator rhod-2 can be used to selectively buffer the bradykinin (Bk)-induced increase in [Ca\(^{2+}\)]\(_m\) and, as a consequence, [ATP\(_m\)] and that this effect was paralleled by a significant decrease in the rate of cytosolic Ca\(^{2+}\) removal.

MATERIALS AND METHODS

Chemicals. Culture material was obtained from Invitrogen (Breda, The Netherlands) and fluorescent dyes were from Molecular Probes (Leiden, The Netherlands). Cellfectin, competent DH10bac Escherichia coli cells, and enzymes for DNA cloning were purchased from Invitrogen. All other reagents were from Sigma (St. Louis, MO).

Cell culture. Fibroblasts of two healthy subjects and two patients in whom an isolated complex I deficiency had been confirmed in both muscle tissue and cultured fibroblasts were derived from a skin biopsy after informed parental consent. The patient cells carried a homozygous missense mutation in either the nuclear gene (C316T; see Ref. 6) or the nuclear NDUFS4 gene (G364A; see Ref. 31) or the nuclear NDUFS4 gene (C316T; see Ref. 6). Cells were cultured in medium 199 (M199) containing 5 mg/ml TWEEN 20, 10% (vol/vol) FCS, 100 IU/ml penicillin, and 100 IU/ml streptomycin.

Baculovirus-mediated expression of mitochondria-targeted aequorin and luciferase in human skin fibroblasts. The baculovirus expression system, which is normally used for protein production in Spodoptera frugiperda 9 insect cells, was made suitable for protein expression in mammalian cells by first removing the herpes simplex virus thymidine kinase polyadenylation signal from the pFastBacDual vector (Invitrogen) with restriction enzymes AccI and XhoI. Next, the p10- and polyhedron promoter were removed from the vector with SmaI and XhoI and replaced with the coding region of a cyanomelagovirus (CMV) promoter digested from the pcDNA1 vector (Invitrogen) with NruI and XhoI. Finally, the cDNA of mitochondria-targeted wild-type aequorin was digested from the AdCMVmLuc vector (1) with XhoI and XbaI and ligated behind the CMV promoter in the modified baculovirus transfer vector (BVTVMaQ). Similarly, the cDNA of mitochondria-targeted luciferase was digested from the AdCMVmLuc vector (1) with EcoRI and XbaI and ligated in the modified vector (BVTVMaL). Competent DH10bac E. coli cells, harboring the baculovirus genome (bacmid) and a transposition helper vector, were transformed with BVTVMaQ or BVTVMaL. Recombinant bacmids, formed upon Tn7-mediated site-specific transposition (17), were isolated and used for transfection of SF9 insect cells with Cellfectin. After 3 days, recombinant baculoviruses were harvested and used to infect SF9 cells at a multiplicity of infection of 0.1. After infection (4 days), the amplified viruses were harvested. To express mitochondria-targeted aequorin or luciferase in human skin fibroblasts, ~20,000 cells were seeded on a glass cover slip (~14 mm) and cultured for 4 h. Cells were then infected with the appropriate virus (5% vol/vol) and cultured in the presence of 1.75 mM sodium butyrate for another 48 h.

Luminescence monitoring of mitochondrial Ca\(^{2+}\) and ATP levels. Before mitochondrial Ca\(^{2+}\) measurements, wild-type aequorin was reconstituted with 5 µM native coelenterazine (Promega, Madison, WI) in serum-free M199 for 1 h at 37°C. Next, the cover slip was placed in the thermostated (37°C) luminometer and perfused (3 ml/min) with HEPES-Tris medium (in mM: 132 NaCl, 4.2 KCl, 1 MgCl\(_2\), 5.5 d-glucose, 10 HEPES, and 1 CaCl\(_2\), pH 7.4). Aequorin luminescence was monitored continuously using a custom-built setup that consisted of a light-shielded low-noise photomultiplier tube (PMT) with a built-in H7360–1 amplifier-discriminator (Hamamatsu Photonics, Shizuoka-Ken, Japan). PMT output was monitored in time using a PCI-6601 photon counting board (National Instruments, Austin, TX) coupled to an IBM-compatible computer using custom-written software (Drs. S. P. Srinivas and W. van Driessche, Laboratory of Physiology, K. U. Leuven, Leuven, Belgium). Light output was integrated during 1 s. At the end of each measurement, signals were calibrated by lysing the cells with 100 µM digitonin in the presence of 10 mM CaCl\(_2\) to destain objective. The fura-2 and aequorin photon emission was converted off-line into [Ca\(^{2+}\)]\(_m\) values, using a computer algorithm described previously (4). To monitor luciferase luminescence with the same system, cells were perfused with HEPES-Tris medium containing 5 µM beetle luciferin (Promega) at 37°C. Light output was recorded at 2-s intervals after which the traces were smoothed off-line using a three-point moving average (OriginPro 6.1; OriginLab, Northampton, MA). Typically, light output from a cover slip of fibroblasts expressing mitochondrial luciferase was 500–1,500 counts/s with a background of 15 counts/s.

Digital imaging microscopy of [Ca\(^{2+}\)]\(_c\) and [Ca\(^{2+}\)]\(_m\). To simultaneously monitor changes in [Ca\(^{2+}\)]\(_c\) and [Ca\(^{2+}\)]\(_m\), fibroblasts, seeded on a glass cover slip (~24 mm), were coloaded with 3 µM fura-2 AM and 5 µM rhod-2 AM, rhod-5F AM, or rhod-FF AM in serum-free M199 for 25 min at 37°C. After loading, cells were washed two times and allowed to equilibrate for another 10 min. Next, cover slips were mounted in a temperature-controlled (37°C) superfusion chamber attached to the stage of an inverted microscope (Axiovert 200 M; Zeiss, Jena, Germany) equipped with a x63, 1.25 numeric aperture (NA) Plan Neofluar objective. The fura-2 and rhodamine dyes were excited at 380 and 540 nm, respectively, using a monochromator (Polychrome IV; TILL Photonics, Gräfelfing, Germany). Fluorescence emission light was directed by a 560DRLP dichroic mirror (Omega Optical, Brattleboro, VT) through a 560DRLP emission filter (Omega) on a CoolSNAP HQ monochrome charge-coupled device (CCD) camera (Roper Scientific, Vianen, The Netherlands). The camera exposure time was set at 200 ms with an interframe interval of 4 s. For ratiometric measurement of [Ca\(^{2+}\)]\(_c\), fibroblasts loaded with fura-2 were alternately excited at 340 and 380 nm via a ×40, 1.3 NA Fluor objective. Fluorescence emission light was directed by a 415 DCLP dichroic mirror (Omega) through a 510WB40 emission filter (Omega) on the CCD camera (camera exposure time of 200 ms and interframe interval of 1 s). At the end of each measurement, cells were scraped off the cover slip to correct for background fluorescence. The kinetics with which the fluorescence emission ratio (R) returned to basal levels was fitted to a monoexponential equation: R(t) = R(0) × e\(^{-t/\tau}\) + R(P), where \(\tau\) is the time constant (in s) and R(P) is the poststimulatory level to which R declines. From a the half-time (\(t_{1/2}\)) was calculated using the equation: \(t_{1/2} = \ln(0.5) \cdot \tau\). All hardware was controlled with Metafluor 6.0 software (Universal Imaging, Downingtown, PA).

Digital imaging microscopy of cellular NADH levels. For NADH measurements, cover slips were mounted in the temperature-controlled (37°C) superfusion chamber of the Axiovert 200 M inverted microscope. Cells were excited at 360 nm, and fluorescence emission light was directed by a 415 DCLP dichroic mirror (Omega) through a 480AF30 emission filter (Omega) on the CCD camera (camera exposure time of 1,000 ms and interframe interval of 4 s).
Data analysis. Numerical data were visualized using Origin Pro 6.1 (OriginLab), and values from multiple experiments were expressed as averages ± SE. Statistical significances were assessed by Student’s t-test.

RESULTS

Changes in mitochondrial and nuclear Ca\(^{2+}\) concentration reported by the rhodamine-based fluorescent Ca\(^{2+}\) indicator rhod-2. Rhodamine-based fluorescent Ca\(^{2+}\) indicators such as rhod-2, rhod-5F, and rhod-FF have a net positive charge and therefore readily sequestrate in the mitochondrial matrix (20). With the final aim of using these indicators as intramitochondrial Ca\(^{2+}\) buffers to assess the relationship between Ca\(^{2+}\)-stimulated mitochondrial ATP production and rate of cytosolic Ca\(^{2+}\) removal, we first evaluated their properties in our cell of interest, the human skin fibroblast. Fibroblasts obtained from a healthy control subject (C1; see Ref. 33) were loaded with 5 μM rhod-2 AM for 25 min at 37°C and subsequently subjected to digital imaging microscopy. In the absence of any stimulus, the cells showed only faint background fluorescence (Fig. 1A, left). At 60 s, cells were stimulated with a maximal concentration of 1 μM Bk. The second left image, taken at 4 s after the onset of stimulation, shows the rapid appearance of fluorescent tubules. The same tubular staining pattern was observed with rhod-5F and rhod-FF. Control experiments employing mitochondria targeted EYFP (COX-EYFP), introduced in these cells by means of the baculovirus expression system, confirmed the mitochondrial nature of these tubules (data not shown).

To monitor the effect of Bk on the free [Ca\(^{2+}\)]\(_{\text{M}}\), we analyzed the change in rhod-2 fluorescence in randomly chosen individual mitochondrial structures in at least two different cells (Fig. 1A). For comparison, the cells were coloaded with the cytosolic Ca\(^{2+}\) indicator fura-2. When [Ca\(^{2+}\)]\(_{\text{C}}\) increases,
the latter indicator displays a decrease in fluorescence signal after excitation at 380 nm. The traces depicted in Fig. 1B show that BK evoked an instantaneous change in both cytosolic (fura-2) and mitochondrial (rhod-2) fluorescence. The rhod-2 signal peaked only few seconds after the fura-2 signal, which reached its highest point at \(-20\) s after the onset of stimulation. The maximum increase in rhod-2 fluorescence was \(5.7 \pm 0.4\) times the prestimulatory value \((n = 10\) mitochondria in 2 cells\). Thereafter, the rhod-2 signal more gradually decreased again to prestimulatory levels reached at 450 s and more after addition of BK. This decrease was fitted monoexponentially with a \(t_{\frac{1}{2}}\) of \(74 \pm 5\) s \((n = 10\) mitochondria in 2 cells\), suggesting the involvement of one major Ca\(^{2+}\) removal process.

The inhibitor of mitochondrial Na\(^+/Ca^{2+}\) exchange, CGP-37157 \((1 \mu M, 2\) min\), did not alter the amplitude of the rhod-2 signal \((5.6 \pm 0.3\) times the prestimulatory value, \(n = 18\) mitochondria in 4 untreated cells vs. \(5.9 \pm 0.2\) times the prestimulatory value, \(n = 24\) mitochondria in 5 CGP-37157-treated cells\). However, the inhibitor significantly reduced the rate of fluorescence decrease \((t_{\frac{1}{2}}\) of \(74 \pm 5\) s, \(n = 18\) mitochondria in 4 untreated cells vs. \(101 \pm 7\) s, \(n = 24\) mitochondria in 5 CGP-37157-treated cells; \(P < 0.01\)).

Rhod-2 did not reside exclusively in the mitochondrial matrix as was indicated by a simultaneous increase in fluorescence in the nucleoplasm (Fig. 1A). The nucleoplasm does not contain mitochondrial structures and, because measurements with fura-2 showed no kinetic differences between the nuclear and cytosolic signal, this compartment was chosen to evaluate the cytosolic properties of rhod-2. Figure 1B shows in contrast to the mitochondrial rhod-2 signal that in the nucleoplasm rhod-2 mirrored the fura-2 signal in this compartment to reach prestimulatory levels already at \(\sim 120\) s after addition of BK.

Changes in \([Ca^{2+}]_{M}\) reported by mitochondria targeted wild-type aequorin. We previously showed that BK \((1 \mu M)\) evoked a rapid increase in luminescence in human skin fibroblasts expressing mitochondria-targeted wild-type aequorin (32, 33). Figure 1C shows that BK virtually instantaneously increased \([Ca^{2+}]_{M}\) to reach a maximum of \(4.6 \pm 0.4\) \(\mu M\) \((n = 7\) cover slips\) after which it rapidly declined again to prestimulatory levels \((t_{\frac{1}{2}}\) of \(6.2 \pm 0.3\) s; \(n = 7\) coverslips\). The duration of the mitochondrial aequorin signal was \(\sim 50\) s, which was considerably shorter than that of the signal obtained with rhod-2 \((450\) s and more\). CGP-37157 \((1 \mu M, 2\) min\) did not alter the peak Ca\(^{2+}\) concentration increase in this compartment \((4.4 \pm 0.1\) \(\mu M, n = 5\) coverslips\). However, whereas the inhibitor markedly slowed down the decrease in rhod-2 fluorescence, it only slightly affected the decrease in aequorin luminescence \((t_{\frac{1}{2}}\) of \(8.1 \pm 0.3\) s, \(n = 6\) coverslips; \(P < 0.05\)). Most probably, the relatively fast decline of the mitochondrial aequorin signal reflects rapid exhaustion of the Ca\(^{2+}\)-sensitive aequorin-coelenterazine complex. When BK was applied to cells pretreated with the protonophore carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; \(1 \mu M, 2\) min), no change in luminescence signal was observed (data not shown), thus demonstrating the mitochondrial localization of the photoprotein (21).

Rhod-2 reduces BK-induced increase in \([Ca^{2+}]_{M}\). Because rhodamine-based Ca\(^{2+}\) indicators preferentially sequestrate in the mitochondrial matrix, we investigated their potential as Ca\(^{2+}\) buffer in this compartment. Healthy fibroblasts (C1; see Ref. 33) expressing mitochondria-targeted aequorin were incubated in the presence of rhod-2 AM \((5\) and \(30\) \(\mu M)\) for \(25\) min at \(37^\circ C\), thoroughly washed, and used for luminescence measurement of the BK-induced increase in \([Ca^{2+}]_{M}\). Figure 2A shows that rhod-2 markedly lowered the amplitude of the BK-induced increase in \([Ca^{2+}]_{M}\). Similarly, rhod-FF \((5\) \(\mu M)\) decreased this amplitude to \(2.5 \pm 0.1\) \(\mu M\) \((n = 6\) coverslips, \(P < 0.01\)).

The effect of rhod-2 was dose dependent, and half-maximal and maximal (80%) reduction of the BK-induced peak increase in \([Ca^{2+}]_{M}\) was obtained with \(3\) and \(30\) \(\mu M\) rhod-2 AM, respectively (Fig. 2B). As a control, we determined the effect of the rhodamine-based indicator for the mitochondrial membrane potential, tetramethylrhodamine methyl ester perchlorate (TMRM). In contrast to rhod-2, TMRM reduced the amplitude of the increase in \([Ca^{2+}]_{M}\) by only 20%. Importantly, cells loaded with \(30\) \(\mu M\) rhod-2 AM displayed a normal BK-induced increase in mitochondrial rhod-2 fluorescence (data not shown), indicating that rhod-2 loading does not interfere with the process of Ca\(^{2+}\) entry in this compartment. These findings demonstrate that rhodamine-based Ca\(^{2+}\) indicators can be used to buffer agonist-induced increases in \([Ca^{2+}]_{M}\).

Rhod-2 inhibits the BK-induced increase in \([ATP]_{M}\). Next, we investigated the effect of rhod-2 on the BK-stimulated increase in \([ATP]_{M}\) in healthy fibroblasts (C1; see Ref. 33) expressing mitochondria-targeted luciferase. Figure 2C shows that BK significantly increased the luminescence signal only after a lag time of \(\sim 22\) s. The rhod-2 markedly reduced the amplitude of the BK-induced increase in \([ATP]_{M}\) without altering, however, its onset and/or duration. The same result was obtained in a single experiment testing two concentrations of rhod-FF (data not shown).

Rhod-2 dose dependently lowered the BK-induced peak increase in luciferase luminescence, and half-maximal and maximal (90%) reduction was achieved with \(3\) and \(30\) \(\mu M\) rhod-2 AM, respectively (Fig. 2D). Pretreatment with rhod-2 AM did not alter the prestimulatory luminescence signal (data not shown), indicating that, unlike FCCP \((1 \mu M, 2\) min; see Ref. 32), the dye did not alter basal mitochondrial ATP levels. TMRM did not affect the luciferase signal, demonstrating that a small (20%) decrease in amplitude of the BK-induced increase in \([ATP]_{M}\) does not lead to a reduction in BK-stimulated mitochondrial ATP production. Similarly to TMRM, rhodamine 123 \((30\) \(\mu M, 25\) min) did not affect the BK-induced increase in \([ATP]_{M}\) (data not shown).

Rhod-2 inhibits the BK-induced increase in NADH fluorescence. Ca\(^{2+}\)-mobilizing agonists have been demonstrated to cause a rapid increase in NADH fluorescence (11). Figure 2E shows that healthy fibroblasts (C1; see Ref. 33) responded with a marked increase in NADH fluorescence after a lag time of \(\sim 12\) s after the addition of BK \((1 \mu M)\). Pretreatment with rhod-2 AM \((30\) \(\mu M, 25\) min) abolished the BK-induced increase in NADH. Of note, rhod-2 did not affect the prestimulatory NADH signal. After background correction, gray values obtained with untreated and rhod-2-treated cells were \(41 \pm 3\) \((n = 9\) cells\) and \(38 \pm 2\) \((n = 9\) cells\), respectively.

Taken together, the above data show that BK \((1 \mu M)\) virtually instantaneously increases \([Ca^{2+}]_{M}\), followed after \(\sim 12\) s by an increase in NADH and after \(\sim 22\) s by an increase in \([ATP]_{M}\). Moreover, they show that rhod-2 AM, when applied at a concentration of \(30\) \(\mu M\) for \(25\) min, dramatically reduces...
the Bk-induced increase in \([\text{Ca}^{2+}]_{\text{M}}\), eradicates the Bk-induced increase in NADH, and virtually abolishes the Bk-induced increase in \([\text{ATP}]_{\text{M}}\).

CGP-37157 fails to restore the inhibitory effect of rhod-2 on the Bk-induced increases in \([\text{Ca}^{2+}]_{\text{M}}\) and \([\text{ATP}]_{\text{M}}\). Previously, we demonstrated that CGP-37157 (1 \(\mu\text{M}, 2\) min) fully restored the Bk-induced increases in \([\text{Ca}^{2+}]_{\text{M}}\) and \([\text{ATP}]_{\text{M}}\) in skin fibroblasts of a patient with a decreased activity of the first complex (complex I) of the electron transport chain without altering these responses in fibroblasts of a healthy control subject (32). Here we show that this drug failed to normalize these two parameters in healthy fibroblasts loaded with 5 \(\mu\text{M}\) rhod-2 (Fig. 3, A and B). This finding is consistent with rhod-2 acting as a \(\text{Ca}^{2+}\) buffer in the mitochondrial matrix.

**Effects of rhod-2 on the Bk-induced increases in \([\text{Ca}^{2+}]_{\text{M}}\) and \([\text{ATP}]_{\text{M}}\) in complex I-deficient patient fibroblasts.** We showed before that the amplitude of the Bk (1 \(\mu\text{M}\))-induced increases in \([\text{Ca}^{2+}]_{\text{M}}\) and \([\text{ATP}]_{\text{M}}\) in skin fibroblasts of a patient with a decreased activity of the first complex (complex I) of the electron transport chain without altering these responses in fibroblasts of a healthy control subject (32). Here we show that this drug failed to normalize these two parameters in healthy fibroblasts loaded with 5 \(\mu\text{M}\) rhod-2 (Fig. 3, A and B). This finding is consistent with rhod-2 acting as a \(\text{Ca}^{2+}\) buffer in the mitochondrial matrix.
Figure 3. CGP-37157 fails to restore the inhibitory effect of rhod-2 on the bradykinin-induced increases in \([Ca^{2+}]_M\) and \([ATP]_M\). A: effect of CGP-37157 (1 \(\mu M\), 2 min) on the bradykinin-induced peak increase in \([Ca^{2+}]_M\) in healthy fibroblasts (C1; see Ref. 33) treated with 5 \(\mu M\) rhod-2 AM. The data presented are averages ± SE of 5–7 coverslips. B: effect of CGP-37157 (1 \(\mu M\), 2 min) on the bradykinin-induced peak increase in \([ATP]_M\) in healthy fibroblasts treated with 5 \(\mu M\) rhod-2 AM. The data presented are averages ± SE of 3–4 cover slips. *Significantly \((P < 0.001)\) different from untreated healthy control cells.

Figure 4. Rhod-2 further reduces the bradykinin-induced increases in \([Ca^{2+}]_M\) and \([ATP]_M\) in complex I-deficient patient fibroblasts. A: rhod-2 (5 \(\mu M\), 25 min) further reduced the bradykinin-induced peak increase in \([Ca^{2+}]_M\) in fibroblasts of a patient with an isolated complex I deficiency associated with a mutation in the nuclear NDUFS7 gene (P11; see Ref. 33) or the nuclear NDUFS4 gene (P9; see Ref. 33). In contrast, the dye did not act as a Ca^{2+} buffer in the cytosolic compartment in C1 cells. In each experiment, both the amplitude of the Bk-induced increase in \([Ca^{2+}]_C\) and the rate at which \([Ca^{2+}]_C\) was subsequently removed from the cytosolic compartment in C1 cells was set at 100%, to which all other values were related.

Neither the amplitude of the Bk-induced increase in \([Ca^{2+}]_C\) nor the rate at which \([Ca^{2+}]_C\) was subsequently removed from the cytosolic compartment differed between C1 and C3 fibroblasts (Table 1). Rhod-2 (5 \(\mu M\)) did not alter the amplitude of the Bk-induced increase in \([Ca^{2+}]_C\) in these healthy control cells. Importantly, the lack of effect of a relatively high concentration of rhod-2 (30 \(\mu M\)) on this parameter demonstrates that the dye did not act as a Ca^{2+} buffer in the cytosolic compartment. In C1 cells, the maximal effect of rhod-2 on the rate of cytosolic Ca^{2+} removal was already obtained after treatment with 5 \(\mu M\) rhod-2 AM. Also in C3 cells this treatment caused a significant decrease in cytosolic Ca^{2+} removal rate. As reported before (32), CGP-37157 (1 \(\mu M\), 2 min) neither changed the peak increase in \([Ca^{2+}]_C\) nor the cytosolic Ca^{2+} removal rate in healthy fibroblasts. Here we show that CGP-37157 failed to reverse the inhibitory effect of rhod-2 (5 \(\mu M\)) on the rate of cytosolic Ca^{2+} removal in these cells.

Reduction in mitochondrial ATP production. Fibroblasts of two healthy control subjects (C1 and C3; see Ref. 33) and two complex I-deficient patients with a mutation in either the nuclear NDUFS7 gene (P11; see Ref. 33) or the nuclear NDUFS4 gene (P9; see Ref. 33) were treated with 5 or 30 \(\mu M\) rhod-2 AM for 25 min at 37°C. In addition, the cells were loaded with fura-2 to monitor the Bk-induced increase in \([Ca^{2+}]_C\). To exclude possible effects of Ca^{2+} influx, the measurements were performed in the absence of extracellular Ca^{2+} (no Ca^{2+} added and 0.5 mM EGTA present).

Figure 5 shows that rhod-2 (5 \(\mu M\)) did not alter the amplitude of the Bk-induced increase in \([Ca^{2+}]_C\) in C1 fibroblasts. On the other hand, the dye significantly slowed down the rate of \([Ca^{2+}]_C\) decrease in these cells. In each experiment, both the amplitude of the Bk-induced increase in \([Ca^{2+}]_C\) and the rate at which \([Ca^{2+}]_C\) was subsequently removed from the cytosolic compartment in C1 cells was set at 100%, to which all other values were related.

The lack of effect of a relatively high concentration of rhod-2 (30 \(\mu M\)) on this parameter demonstrates that the dye did not act as a Ca^{2+} buffer in the cytosolic compartment. In C1 cells, the maximal effect of rhod-2 on the rate of cytosolic Ca^{2+} removal was already obtained after treatment with 5 \(\mu M\) rhod-2 AM. Also in C3 cells this treatment caused a significant decrease in cytosolic Ca^{2+} removal rate. As reported before (32), CGP-37157 (1 \(\mu M\), 2 min) neither changed the peak increase in \([Ca^{2+}]_C\) nor the cytosolic Ca^{2+} removal rate in healthy fibroblasts. Here we show that CGP-37157 failed to reverse the inhibitory effect of rhod-2 (5 \(\mu M\)) on the rate of cytosolic Ca^{2+} removal in these cells.

*Significantly \((P < 0.001)\) different from untreated healthy control cells. *Significantly \((P < 0.05)\) different from untreated patient cells. *Significantly \((P < 0.01)\) different from untreated healthy control cells.
Fig. 5. Rhod-2 decreases the rate of cytosolic Ca\(^{2+}\) removal. Fibroblasts coloaded with 3 and 5 \(\mu\)M rhod-2 AM were alternately excited at 340 and 380 nm for digital imaging microscopy of the bradykinin (1 \(\mu\)M)-induced changes in fura-2 fluorescence in the cytosolic compartment. Measurements were performed in the absence of extracellular Ca\(^{2+}\) (no Ca\(^{2+}\) added and 0.5 mM EGTA present). The rhod-2 slowed down the rate of cytosolic Ca\(^{2+}\) removal but had no effect on the peak Ca\(^{2+}\) concentration increase in this compartment (2 representative traces).

Both the amplitude of the Bk-induced increase in [Ca\(^{2+}\)]\(_C\) and the rate of cytosolic Ca\(^{2+}\) removal were significantly reduced in patient fibroblasts (Table 1). In P11 cells, neither 5 nor 30 \(\mu\)M rhod-2 caused any further reduction of these two parameters. The same observation was reached with 5 \(\mu\)M rhod-2 in P9 fibroblasts. CGP-37157 (1 \(\mu\)M, 2 min) normalized both the Bk-induced increase in [Ca\(^{2+}\)]\(_C\) and the rate of cytosolic Ca\(^{2+}\) removal in patient fibroblasts. Rhod-2 did not prevent restoration of the Bk-induced increase in [Ca\(^{2+}\)]\(_C\) by CGP-37157 in these cells. On the other hand, it abolished normalization of the rate of cytosolic Ca\(^{2+}\) removal by this drug.

**DISCUSSION**

The data presented in this study demonstrate that cytosolic Ca\(^{2+}\) removal is significantly accelerated as a consequence of increased mitochondrial ATP production in cells stimulated with a Ca\(^{2+}\)-mobilizing agonist. Evidence comes from the observation that both processes were markedly reduced in human skin fibroblasts of healthy subjects loaded with the rhodamine-based fluorescent Ca\(^{2+}\) indicator rhod-2. Because of its positive charge, this indicator readily sequestered in the mitochondrial matrix, where it reduced the Bk-induced increase in Ca\(^{2+}\) and ATP concentration in a dose-dependent manner. Of crucial importance, fura-2 measurements revealed that, even at a relatively high concentration of 30 \(\mu\)M, rhod-2 did not alter the Bk-induced increase in [Ca\(^{2+}\)]\(_C\), demonstrating that the dye did not act as a Ca\(^{2+}\) buffer in the cytosolic compartment. Taken together, the present findings are compatible with the idea that Bk virtually instantaneously increases [Ca\(^{2+}\)]\(_C\) and, as a consequence, [Ca\(^{2+}\)]\(_M\) to boost the production of NADH (after a lag time of ~12 s) and subsequently ATP (after a lag time of ~22 s), which is then released in the cytosol, where it accelerates the Ca\(^{2+}\) removal process.

In the absence of external Ca\(^{2+}\), the complex I-deficient patient fibroblasts used in this study displayed a cytosolic Ca\(^{2+}\) removal rate that was ~40% lower than that of healthy fibroblasts (Table 1). Of note, these patient fibroblasts consistently showed an ~60% reduction in cytosolic Ca\(^{2+}\) removal rate when stimulated in the presence of external Ca\(^{2+}\) (32, 33). This extent of reduction in Ca\(^{2+}\) removal rate was found to be paralleled by a 50% decrease in Bk-stimulated mitochondrial ATP production (32, 33). Surprisingly, further inhibition of the Bk-induced increase in [Ca\(^{2+}\)]\(_M\) and [ATP]\(_M\) by rhod-2 (5 \(\mu\)M) did not decrease the cytosolic Ca\(^{2+}\) removal rate in these patient cells. Similarly, in rhod-2-treated healthy fibroblasts, the cytosolic Ca\(^{2+}\) removal rate was already maximally decreased by 20% when the Bk-induced increase in [ATP]\(_M\) was only partially (60%) inhibited. Together, these findings indicate that cytosolic Ca\(^{2+}\) removal is accelerated only when the Ca\(^{2+}\)-stimulated increase in [ATP]\(_M\) exceeds 50–60% of its maximum. Notably, the lack of effect of rhod-2 on the rate of cytosolic Ca\(^{2+}\) removal in patient fibroblasts demonstrates that the dye does not interfere with this process other than by inhibiting Ca\(^{2+}\)-stimulated mitochondrial ATP production. The reduced rate of cytosolic Ca\(^{2+}\) removal observed in patient fibroblasts was completely restored upon normalization of the Ca\(^{2+}\)-stimulated increase in mitochondrial ATP production by CGP-37157, an inhibitor of mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange (5, 16, 32). This finding demonstrates that the decrease in Ca\(^{2+}\) removal rate observed in complex I-deficient patient fibroblasts is the result of a reduced ATP supply to the cytosolic Ca\(^{2+}\) pumps rather than a pathological decrease in their amount and/or change in geometry of the Ca\(^{2+}\)-handling

**Table 1. Effects of rhod-2 and CGP-37157 on the Bk-induced changes in [Ca\(^{2+}\)]\(_C\) in fibroblasts of healthy control subjects and patients with an isolated complex I deficiency**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Not Pretreated</th>
<th>Rhod-2 (5 (\mu)M)</th>
<th>Rhod-2 (30 (\mu)M)</th>
<th>CGP-37157 (1 (\mu)M)</th>
<th>Rhod-2 (5 (\mu)M) and CGP-37157 (1 (\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1 (no. 5120)</td>
<td>100±1 (109) 100±2</td>
<td>102±2 (85) 122±2*</td>
<td>98±2 (57) 123±2*</td>
<td>105±3 (65) 97±2</td>
<td>99±2 (59) 122±3*</td>
</tr>
<tr>
<td>C3 (no. 5119)</td>
<td>107±1 (20) 98±2</td>
<td>97±6 (23) 118±3*</td>
<td>104±7 (19) 98±2</td>
<td>106±6 (28) 98±2</td>
<td>96±8 (28) 138±3*</td>
</tr>
<tr>
<td>P9 (no. 5260)</td>
<td>81±6* (30) 136±3*</td>
<td>79±5 (22) 135±3*</td>
<td>86±1* (41) 147±4*</td>
<td>98±2 (33) 140±4</td>
<td>99±2 (44) 144±4*</td>
</tr>
</tbody>
</table>

Table values are means ± SE; nos. in parentheses indicate the no. of cells. Peak [Ca\(^{2+}\)]\(_C\), maximal amplitude of the Bk-induced increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_C\)); \(r_v\) [Ca\(^{2+}\)]\(_C\), half-time of the decay phase of the Bk-induced [Ca\(^{2+}\)]\(_C\) transient. Fibroblasts, coloaded with 3 \(\mu\)M fura-2 AM and 5 or 30 \(\mu\)M rhod-2 AM (25 min, 37°C) were alternately excited at 340 and 380 nm for digital imaging microscopy of the bradykinin (1 \(\mu\)M)-induced changes in fura-2 fluorescence in the cytosolic compartment. Measurements were performed in the absence of extracellular Ca\(^{2+}\) (no Ca\(^{2+}\) added and 0.5 mM EGTA present). All values are expressed as percentage of the value obtained with C1 cells in the same experiment. *Values significantly (P < 0.05) different from untreated C1 cells (33).
compartments. The fact that patient fibroblasts displayed a cytosolic Ca\(^{2+}\) removal rate that was markedly (20%) lower than that of healthy fibroblasts treated with either rhod-2 (this study) or FCCP (32) may then suggest that ATP supply from sources other than the mitochondria is decreased in patient fibroblasts. It remains to be established whether this involves a pathological decrease in glycolytic activity and/or increase in ATP consumption.

The patients used in this study have been described extensively with respect to clinical presentation, disease course, and results of laboratory studies (6, 31). In both cases, the clinical course was progressive, with first signs of the disease appearing at 4 mo (P9; see Ref. 6) and 11 mo (P11; see Ref. 31) after birth. The children suffered from severe multisystem defects, and death occurred at 8 mo and 5 yr of age, respectively. Extrapolation of our experimental data to the clinical setting suggests that tissues and organs that have high energy demands encounter severe problems with maintaining energy supply, especially under working conditions, and are therefore vulnerable to a gradual loss of integrity. The two patient cell lines investigated here showed major differences in Bk-stimulated mitochondrial ATP production (33) and cytosolic Ca\(^{2+}\) removal rate (Ref. 33 and this study), indicating that the speed of disease progression depends on multiple factors. This is stressed by our finding that 4 out of 14 complex I-deficient patient cell lines displayed a normal Bk-stimulated increase in [ATP]\(_{M}\) (33).

The inability of CGP-37157 to normalize the increase in [Ca\(^{2+}\)]\(_{M}\) in rhod-2-treated healthy fibroblasts is illustrative of the buffering capacity of this mitochondrial Ca\(^ {2+}\) indicator. However, whereas CGP-37157 failed to restore the Ca\(^{2+}\) removal rate in rhod-2-treated patient cells, it fully normalized the peak [Ca\(^{2+}\)]\(_{C}\) increase in these cells. We previously showed that CGP-37157 (1 \(\mu\)M, 2 min) does not increase the ER Ca\(^{2+}\) content in these patient cells (32), thus disfavoring this explanation for the observed normalization of the peak [Ca\(^{2+}\)]\(_{C}\) increase. Another possibility is that CGP-37157 caused saturation of the capacity of the mitochondria to sequester Ca\(^{2+}\). This was demonstrated for the human umbilical vein endothelial cell line EA.hy926 (18). However, the present finding that rhod-2, despite its buffering capacity, did not prevent normalization of the peak [Ca\(^{2+}\)]\(_{C}\) increase by CGP-37157 argues against this possibility. Finally, the fact that rhod-2 alone did not augment the peak [Ca\(^{2+}\)]\(_{C}\) increase in these patient fibroblasts argues against a mechanism involving increased mitochondrial Ca\(^{2+}\) uptake. Therefore, CGP-37157 may somehow promote the process of IP\(_3\)-stimulated Ca\(^{2+}\) release from the ER.

We previously showed that the Bk-induced increase in [Ca\(^{2+}\)]\(_{C}\) was significantly reduced in complex I-deficient patient fibroblasts (32, 33). As mentioned already, this decrease was found to be paralleled by a decrease in ER Ca\(^{2+}\) content. This finding is in agreement with the above conclusion that ATP supply to cytosolic Ca\(^{2+}\) pumps such as the SERCA is reduced in patient fibroblasts. The present study shows that rhod-2 treatment did not affect the Bk-induced increase in [Ca\(^{2+}\)]\(_{C}\) in control and patient fibroblasts, indicating that the ER Ca\(^{2+}\) content was not altered by the presence of the Ca\(^{2+}\) buffer in the mitochondrial matrix.

In fibroblasts of a healthy control subject, rhod-2 maximally reduced the cytosolic Ca\(^{2+}\) removal rate by 20%. The same percentage of inhibition was observed after dissipation of the mitochondrial membrane potential by means of FCCP (1 \(\mu\)M; 2 min; see Ref. 32). This is remarkable because FCCP caused an immediate drop of the mitochondrial luciferase signal by \(-60\%\), whereas, in sharp contrast, this signal remained invariably high in cells loaded with rhod-2. These findings can be explained by assuming that mitochondria contribute to active cytosolic Ca\(^{2+}\) extrusion only after an increase in [Ca\(^{2+}\)]\(_{M}\), which not only boosts ATP production but also triggers ADP/ ATP exchange. According to this model, rhod-2 will inhibit both the Ca\(^ {2+}\)-induced production and export of ATP. Alternatively, the finding that Bk evokes a relatively small (30%) increase in average mitochondrial ATP signal may suggest that Ca\(^{2+}\)-mobilizing agonists increase [ATP]\(_{M}\) only in a relatively small subset of mitochondria closely juxtaposed to agonist-sensitive Ca\(^{2+}\) release sites at the ER (22–24; also see Ref. 10). Accordingly, rhod-2 will prevent the Ca\(^{2+}\)-induced production of ATP in these mitochondria, while leaving the ATP levels in agonist-insensitive mitochondria intact.

Evidence has been provided that Ca\(^{2+}\) on the external side of the inner mitochondrial membrane can regulate mitochondrial ATP production by activating the aspartate-malate NADH shuttle (15, 19). Thus far, however, the role of Ca\(^{2+}\) in agonist-stimulated mitochondrial ATP production has been addressed solely through manipulation of the agonist-induced increase in [Ca\(^{2+}\)]\(_{C}\) (12). Obviously, this maneuver does not provide information on the relative importance of the Ca\(^{2+}\)-stimulated aspartate-malate NADH shuttle. The present finding that rhod-2 abolished the Bk-induced increases in NADH content (completely) and [ATP]\(_{M}\) (virtually completely) without altering the Bk-induced increase in extramitochondrial [Ca\(^{2+}\)]\(_{C}\) demonstrates that the aspartate-malate NADH shuttle, if activated, requires an additional increase in intramitochondrial [Ca\(^{2+}\)]\(_{C}\) to contribute to agonist-stimulated mitochondrial ATP production in human skin fibroblasts.

Bk readily increased the rhod-2 fluorescence emission intensity in individual mitochondria loaded with 30 \(\mu\)M rhod-2 AM (data not shown). This indicates that high concentrations of rhod-2 do not interfere with Ca\(^{2+}\)-stimulated mitochondrial ATP production through inhibition of the mitochondrial Ca\(^{2+}\) entry pathway. The specificity of rhod-2 was demonstrated by the fact that the rhodamine-based mitochondrial membrane potential indicator TMRM maximally reduced the amplitude of the Bk-induced increase in matrix Ca\(^{2+}\) concentration by only 20% and had no effect on the Bk-induced increase in mitochondrial ATP production. The latter observation is consistent with the finding in HeLa cells that inhibition of histamine-stimulated mitochondrial ATP production occurred only when the amplitude of the agonist-induced increase in matrix Ca\(^{2+}\) concentration was reduced by >20% (12). Similarly, cells treated with 30 \(\mu\)M rhodamine 123 displayed a normal increase in mitochondrial ATP production after stimulation with Bk.

Finally, the data presented show that rhod-2 half-maximally inhibited the Bk-induced increases in [Ca\(^{2+}\)]\(_{M}\) and [ATP]\(_{M}\) in cells loaded with \(-3 \mu\)M rhod-2 AM for 25 min at 37°C. Because most studies use similar loading conditions (3, 6, 9, 11, 25, 29), results should be regarded with care.

GRANTS

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REDUCED CYTOSOLIC Ca\(^{2+}\) REMOVAL IN HUMAN COMPLEX I DEFICIENCY


REFERENCES


