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ESTIMATION OF INTRAMITOCHONDRIAL pCa AND pH BY FURA-2 AND 2,7 BISCARBOXYETHYL-5(6)-CARBOXYFLUORESCEIN (BCECF) FLUORESCENCE

Michael H. Davis, Ruth A. Altschuld, Dennis W. Jung, and Gerald P. Brierley

Department of Physiological Chemistry, Ohio State University Medical Center, Columbus, Ohio 43210

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Isolated heart mitochondria hydrolyze the acetoxymethyl esters of the Ca^{2+}-sensitive fluorescent probe fura-2 and the fluorescent pH indicator biscarboxyethyl-5(6)-carboxyfluorescein (BCECF). The free acid forms of both probes are retained in the matrix and their fluorescence can be used to monitor the pCa and pH, respectively, of this compartment. When fura-2 loaded rat heart myocytes are lysed with digitonin, a portion of the dye is retained in the mitochondrial fraction and its fluorescence reports the uptake and release of Ca^{2+} by the mitochondria. It is concluded that fura-2 and BCECF may report mitochondrial as well as cytosol parameters when the probes are used in intact cells.

The fluorescent Ca^{2+} indicator fura-2 has been used to monitor intracellular free Ca^{2+} in a wide variety of cells (see 1-5, for example). The analogous fluorescent pH indicator BCECF has also been widely used to follow changes in intracellular pH (6-8). With both fura-2 and BCECF, the lipid-soluble acetoxymethyl ester is taken up by the cells and hydrolyzed by cellular esterases to produce the relatively non-permeant free acid. In the present study we report that isolated heart mitochondria can be loaded with fura-2 or BCECF by procedures quite analogous to those used with isolated cells and that the fluorescence of these probes provides a continuous record of intramitochondrial pCa or pH, respectively, under the appropriate conditions. In addition, when isolated adult rat heart myocytes are equilibrated with fura-2 and then lysed with digitonin, a considerable portion of the indicator is localized in the particulate fraction. This fura-2 fluorescence is consistent

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with the uptake and release of Ca^{2+} by the mitochondria in these cells. These results raise the possibility that changes in mitochondrial pCa or pH may contribute to the fluorescence of these probes in intact cells and thereby affect estimates of cytosol free Ca^{2+} or pH.

RESULTS AND DISCUSSION

Uptake of Fura-2 by the Mitochondrial Fraction of Adult Heart Myocytes - When Ca^{2+}-tolerant adult rat heart myocytes are loaded with fura-2 the fluorescence indicates that intracellular free Ca^{2+} is maintained at about 125 nM (Fig. 1A). When these cells

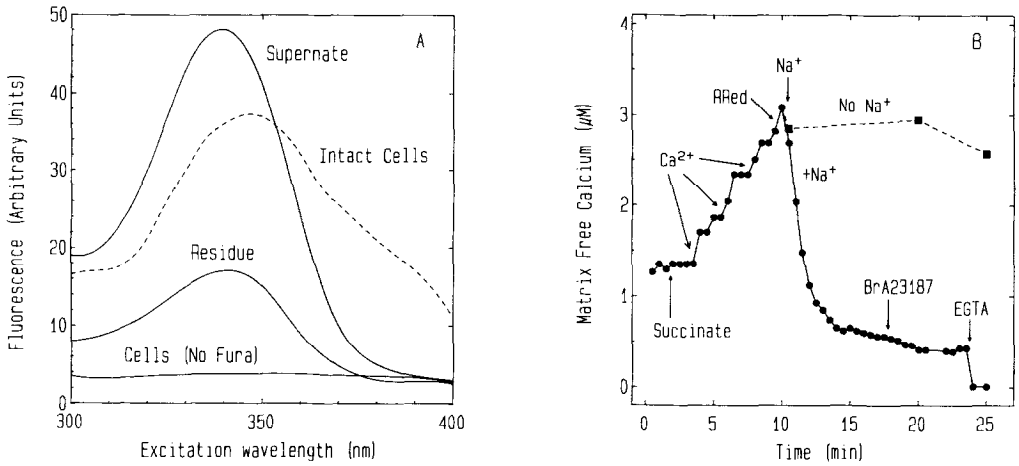

Fig. 1. Retention of fura-2 by the mitochondrial fraction of intact, Ca^{2+}-tolerant rat heart myocytes. A. Excitation spectra of intact fura-2 loaded myocytes and digitonin-lysed myocytes. Myocytes were prepared as described by Altschuld et al (9) and incubated at 1 mg protein/ml in Krebs-Henseleit buffered with HEPES and containing 1 mM Ca^{2+} and 2 μM fura-2 ester. After 40 min at 25°C the cells were washed, resuspended in the same medium without fura-2 and allowed to stand for one hr. Spectra were recorded in a Perkin-Elmer LS-5B fluorimeter with emission at 510 nm. For intact cells at 0.1 mg/ml the ratio of emission at 350/380 nm was converted to free Ca^{2+} concentration as described by Li et al (10). Fura-2 loaded myocytes were lysed with digitonin at 16 μg/mg (11), centrifuged and the digitonin-insoluble residue resuspended in a new change of the suspending medium. Both the digitonin residue and the supernate contain fura-2 saturated with Ca^{2+} under these conditions. B. Uptake and release of Ca^{2+} reported by fura-2 in the mitochondrial fraction of digitonin-lysed myocytes. Myocytes were loaded with fura-2 as in A, suspended in choline chloride (150 mM) containing KCl (5mM) and HEPES buffer (5 mM, pH 7.4), washed once in this medium containing 2 mM EGTA and twice in 50 μM EGTA, treated with rotenone and then lysed with digitonin (11). The lysed cells were suspended in the choline chloride, 50 μM EGTA medium, and fluorescence ratio recorded following addition of succinate 3 mM, 10 μM increments of $CaCl_2$, ruthenium red (R Red, 1μM), ±NaCl (50 mM), Br A23187 (0.3 μM) and EGTA (1 mM).

are treated with digitonin, the sarcolemma is disrupted, but the mitochondria, sarcoplasmic reticulum and contractile elements remain intact and functional (11). Separation of the digitonin-insoluble residue from the suspending medium and cytosol by centrifugation reveals that a significant portion of the Ca^{2+}-responsive fura-2 fluorescence is retained by the particulate fraction (Fig. 1A). Lysis of individual fura-2 loaded myocytes with digitonin in the fluorescence microscope also shows that considerable Ca^{2+}-responsive dye is retained by the cells (Q. Li, to be published).

When fura-2 loaded myocytes are lysed with digitonin in a Ca^{2+}-free medium, washed free of cytosol components and suspended in choline chloride containing EGTA, the addition of small increments of Ca^{2+} (10 μM) results in increases in free Ca^{2+} as reported by fura-2 fluorescence (Fig. 1B). The increase in free Ca^{2+} is respiration-dependent and ruthenium red-sensitive (not shown), and is therefore consistent with the uptake of Ca^{2+} by the mitochondrial fraction of the digitonin-lysed cells. The fura-2 fluorescence also shows a Na^{+}-dependent release of Ca^{2+} from this fraction (Fig. 1B) that is strongly inhibited by diltiazem (not shown). The uptake and release of Ca^{2+} as indicated by fura-2 fluorescence in digitonin-lysed myocytes is therefore quite consistent with the presence of a Ca^{2+}-responsive form of this probe in the mitochondria as the result of loading the probe into intact cells (see also 5). We conclude (in contrast to others, Ref. 1-4, for example) that fura-2 is taken up by mitochondria in intact heart cells and that the fluorescence of this probe may reflect changes in this compartment as well as the cytosol as is usually assumed.

Uptake of Fura-2 by Isolated Heart Mitochondria - When isolated heart mitochondria are incubated with fura-2 ester under conditions analogous to those used to introduce the indicator into intact cells, the mitochondria take up a large proportion of the available dye, retain it during washing and show a typical, Ca^{2+}-responsive excitation spectrum for fura-2 when re-isolated (Fig 2A). The fluorescence of fura-2 loaded mitochondria decreases as endogenous total Ca^{2+} is lost to a medium containing EGTA (Fig. 2B). The fluorescence of fura-2 loaded mitochondria also reports respiration-dependent, ruthenium red-sensitive Ca^{2+} uptake and Na^{+}-dependent, diltiazem-sensitive Ca^{2+} loss as expected in experiments analogous to those of Fig. 1B (see 13 for

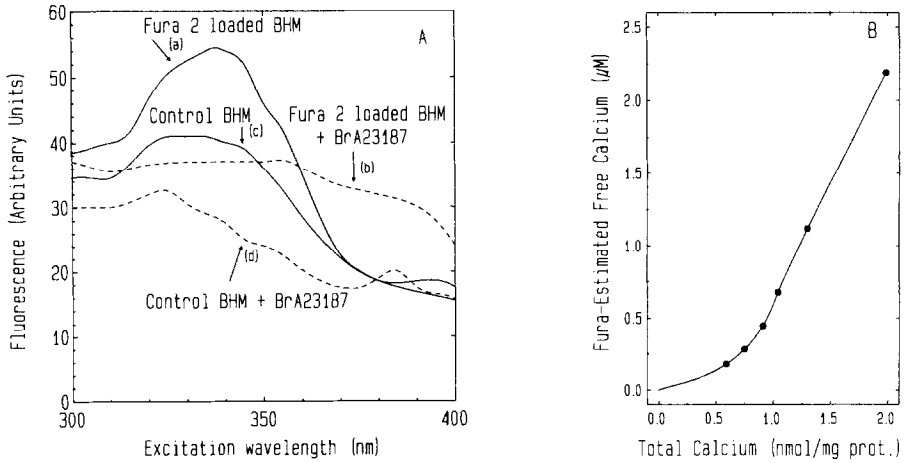

Fig. 2. Uptake of fura-2 by isolated heart mitochondria (A) and the responsiveness of the probe fluorescence to changes in matrix Ca^{2+} (B). A. Beef heart mitochondria, prepared as previously described (12), were incubated at 25 mg protein/ml in buffered 0.25 M sucrose with fura-2 ester (2 μM) for 30 min at 20°C. The mitochondria were centrifuged, washed once and suspended at 0.4 mg/ml in a medium of KCl (100 mM), TES (10 mM, pH 7.2), malate and glutamate (5 mM each). Control mitochondria were carried through all the steps in the absence of fura-2 ester. (a) Fura-2 loaded mitochondria, (b) Fura-2 mitochondria treated with EGTA (2.5 mM) and Bromo A23187 (0.3 μM), (c) Control mitochondria (d) control plus EGTA and BrA23187. B. Fura-2 loaded mitochondria were suspended in the above medium containing EGTA (1 mM). The loss in total Ca^{2+} was determined by atomic absorption as a function of time of incubation and compared with free Ca^{2+} estimated by fura-2 fluorescence (converted to Ca^{2+} concentration as described in Ref. 10).

a review of Ca^{2+} uptake and loss by heart mitochondria). These experiments establish that fura-2 fluorescence can be used to follow changes in mitochondrial free Ca^{2+} when the total Ca^{2+} in the matrix is kept in a low concentration range. A correction must be made (see 4) for the autofluorescence of mitochondria in the absence of fura-2 (Fig. 2A) in these protocols, because its contribution can become significant under conditions that produce changes in the redox state of the NAD(H) pool.

Uptake of BCECF by Heart Mitochondria - Isolated heart mitochondria also take up the acetoxymethyl ester of BCECF and hydrolyze it to the pH-sensitive fluorescent indicator. The free acid is not taken up by mitochondria. As with intact cells (see 6,7), the ratio of fluorescence at a pH-sensitive excitation wavelength (500nm) to that at the non-responsive 450 nm can be calibrated in terms of matrix pH. The matrix pH as reported by BCECF fluorescence shifts from about pH 7.7 to 8.3 when succinate respiration is initiated in a KCl medium (Fig. 3). Addition of

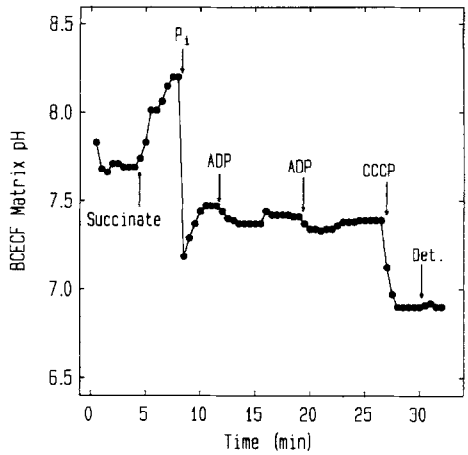

Fig. 3. Intramitochondrial pH during a state 3 to 4 transition as reported by BCECF fluorescence. Beef heart mitochondria (25 mg/ml) were treated with 15 µM BCECF ester for 20 min at 20° in buffered 0.25 M sucrose, centrifuged, washed and suspended at 0.4 mg/ml in a medium of KCl (100 mM), TES (10 mM, pH 6.8), EGTA (30 µM) and rotenone (3 µg/ml). The fluorescence ratio 500/450 was recorded and converted to pH using a standard curve (7). Where indicated K^+ phosphate (2 mM), ADP (60 µM), m-chlorocarbonyl-cyanidephenylhydrazone (CCCP, 1.5 µM) and Triton X-100 (Det.; 0.07%) were added.

P_i causes a rapid acid shift of about one pH unit due to H^+ and P_i symport and addition of ADP produces a transient acidification consistent with the utilization of protonmotive force for ATP synthesis (14). The pH changes reported by BCECF agree qualitatively with those found by Nicholls using acetate distribution in an analogous protocol (Fig. 6 of Ref. 14).

The generation of BCECF from its ester is readily followed by the changes in the excitation spectrum. More than 90% of the ester is trapped in the matrix after incubation of mitochondria (25 mg/ml) for 20 min at 25° with 15 µM ester. The free acid is retained well with less than 10% lost after 5 hrs on ice. The hydrolysis of BCECF ester shows an optimum at pH 8.6, a V_{max} of 0.09 nmol·mg^{-1}·min^{-1} and an apparent K_m of 6µM. The hydrolysis is temperature dependent with an E_a of 18 kcal/mol and inhibited 50 to 60% by uncouplers. This inhibition appears to depend on the pH change associated with uncoupling (see Fig. 3), since it is largely eliminated by nigericin in a KCl medium. Hydrolysis is also inhibited 20 to 25% by butacaine (0.5 mM). The properties of the hydrolysis reaction are consistent with the participation of a rather non-specific lipase or esterase that shows good activity under the conditions found in the matrix of

respiring mitochondria. Brain mitochondria have also been found to hydrolyze the ester of fura-2 under similar conditions (15).

These studies have established that heart mitochondria have considerable capacity to take up and retain fluorescence probes such as fura-2 and BCECF. The presence of these probes in the matrix results in little change in state 3 respiration, ADP:O ratio or acceptor control index and it seems likely that they will be useful for monitoring the pCa or pH of isolated mitochondria.

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