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Calcium Metabolism and Cystic Fibrosis: Mitochondrial Abnormalities Suggest a Modification of the Mitochondrial Membrane

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Summary

A disorder of calcium (Ca^{2+}) metabolism may be central to the pathogenesis of cystic fibrosis (CF). Average cellular Ca^{2+} levels in fibroblasts derived from patients with CF (ages, 14–25 yr; n = 25) were 36–77% higher than in matched controls depending on age of cell culture (9.0–10.6 *versus* 5.1–7.8 nmol/mg cellular protein). Cellular Ca^{2+} was significantly elevated in CF, but was not a reliable criterion for identifying CF cells because of the high variability of results. Studies of Ca^{2+} fluxes in cell organelles showed that mitochondria isolated from CF fibroblasts accumulate 2–3 times more Ca^{2+} than controls [79.5 ± 8.2 *versus* 33.7 ± 4.7 nmols · mg mitochondrial protein^{-1} · 10 min^{-1} (±SD)]. Ca^{2+} accumulation in mitochondria reliably distinguished between CF and control or heterozygote cells ($P < 0.0005$, $n = 11$).

***In vitro* experiments showed that Ca^{2+} influx and efflux are increased in isolated CF mitochondria, resulting in net Ca^{2+} accumulation. Ca^{2+} uptake in mitochondria is energy-dependent; some inhibitors of mitochondrial energy metabolism (atractyloside, oligomycin) influenced Ca^{2+} uptake significantly more in CF than in control mitochondria. Furthermore, the average activities of NADH oxidase, NADH- and succinate-cytochrome c reductase were 77, 58, and 48% higher in CF mitochondria, respectively. This indicates that many functions associated with energy metabolism and the mitochondrial membrane (electron transport, ATP transport, and ATP hydrolysis) are not operating properly in CF, thus possibly causing the derangement of Ca^{2+} metabolism found in CF mitochondria and cells.**

Abbreviation

CF, cystic fibrosis

An interesting feature of CF research is the multiformity of results reported in the literature (8, 33, 34). Only recently, studies of the Vatican records on dispensations for cousin-cousin marriages have confirmed that CF is a *monogenic* autosomal recessive disease with an incidence of up to 1 in 1600 in some Caucasian populations (*i.e.*, northern Europe) (9). Because only a single gene must account for all the phenomenons of CF, it seems reasonable to conceive a working model which would explain this heterogenous exocrinopathy that still is the most common lethal genetic disease in Europe and the United States (8, 35).

CF must surely involve some basic event of cellular function or the diversity of reported results is hardly comprehensible. The emphasis of our studies was put on Ca^{2+} metabolism which has been suggested to be central in the pathogenesis of CF by various authors (2, 3, 16, 18, 28). Ca^{2+}, an intracellular messenger, is linked to many cellular functions (*e.g.*, secretion, protein phosphorylation and activation, and protein synthesis; for a recent review see ref. 7). A derangement, therefore, of cellular Ca^{2+} can cause a variety of different phenomenons, making it a possible candidate for explaining the manifold features reported in CF.

Our study's first aim was to quantify elevated intracellular Ca^{2+} in CF and see if it is possible to establish a simple and reliable test to discriminate between CF and control cells on this basis. Second, Ca^{2+} metabolism was studied in cell organelles (mitochondria, endoplasmic reticulum) that account for 95–98% of intracellular Ca^{2+} and presumably must be affected if intracellular Ca^{2+} is significantly altered. Our investigations centered on the energy-dependent Ca^{2+} uptake and Ca^{2+} efflux in mitochondria which showed to be definitely altered in CF cells. By employing different inhibitors of mitochondrial Ca^{2+} uptake, ATP transport, ATP hydrolysis and electron transfer, and by measuring electron transfer activities directly, changes in mitochondrial Ca^{2+} and energy metabolism could be detected in CF. The suggestion of a mitochondrial focus in CF, first made by Feigal and Shapiro, was confirmed (11, 28).

MATERIALS AND METHODS

Cell cultures. Fibroblast cultures were obtained from biopsy specimens of the forearm, after informed consent had been obtained. Thirty-two patients with CF (14–25 yr; mean, 17.3 yr) were studied. Their clinical status varied from good to severe with Shwachmann scores (31) varying between 34 and 92. Furthermore, 28 obligate CF heterozygotes (21–33 yr; mean, 29.1 yr) and 51 controls (14–30 yr; mean, 18.4 yr) with no signs of pulmonary disease and in good clinical condition were studied.

Controls were sex- and age-matched. Flasks (Falcon), 75 cm^2, or, for mitochondrial preparations, 110 × 465-mm roller bottles (Wheaton) were utilized. Fibroblast monolayers were cultured in Eagles minimum essential medium (GIBCO) containing 10% fetal bovine serum (Boehringer, Mannheim) with 200 U penicillin and 50 μg/ml streptomycin (GIBCO). Cells were grown at 37°C in a humidified 95% air–5% CO_2 atmosphere and were used at the passages indicated. For storage, cells were frozen in liquid nitrogen at early passages and thawed for use as needed. Removal of cells from stock flasks or roller bottles occurred either by trypsin/EDTA (1:500 trypsin with 0.3 g/l EDTA) or by using a rubber scraper as indicated.

Intracellular Ca^{2+} content. Cells were cultured in the presence of 0.1–0.5 mCi [$^{45}Ca^{2+}$] (New England Nuclear, 30 mCi/mg) over different time intervals and then harvested by trypsination. Immediately afterwards, cells were washed once with 0.9% NaCl and resuspended in the same former culture medium (including [$^{45}Ca^{2+}$]) saturated with 95% air–5% CO_2 for 2 h. They were shaken occasionally during this period to avoid adherence. Cells were then washed twice with 3 mM $LaCl_3$, 1 mM EDTA in 0.9% NaCl, pH 7.4 at 0°C to remove external Ca^{2+}. Contamination of external [$^{45}Ca^{2+}$] was determined by adding [$^{45}Ca^{2+}$] to unlabeled cells during washing. It never exceeded 8% of intracellular Ca^{2+}. Uptake experiments over three cell doublings, after which at least 87.5% of total cellular Ca^{2+} must be in equilibrium with [$^{45}Ca^{2+}$] even if no exchange occurs, indicated that the bulk of cellular Ca^{2+} had exchanged with [$^{45}Ca^{2+}$] within 24 h, thus verifying the use of 24-h [$^{45}Ca^{2+}$] incorporation as a monitor of total intracellular Ca^{2+}. The specific [$^{45}Ca^{2+}$] activity count/min ^{45}Ca/mol Ca) was measured by atomic absorption spectrophotometry and liquid scintillation techniques. Protein determinations were done in aliquots of washed cells by the biuret or Lowry method (21).

Mitochondria preparations. Confluent fibroblast monolayers were treated according to a modified method described by Feigal and Shapiro (11). Roller bottles with attached cells were harvested by prolonged trypsination (15–20 min) to yield at least 50 × 10^6 cells. The cells were then washed twice at 4°C with medium A: 200 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 0.1% fatty acid free bovine serum albumin, and 10 mM HEPES, adjusted to pH 7.2. The temperature was maintained at 0–4°C for all following steps until incubation with [$^{45}Ca^{2+}$]. After resuspending the cells in medium A (1:4 v/v), they were homogenized in a tight-fitting Dounce homogenizer with 20–30 strokes. Usually 80–90% of the cells were disrupted, as determined by microscopy. The homogenate was centrifuged at 1500 *g* for 5 min and the supernate was subsequently centrifuged at 16,000 *g* for 15 min to yield a mitochondrial pellet. The low spin pellet was discarded. Mitochondria were then washed once with medium A without EGTA. The homogeneity of mitochondria was checked by electron microscopy. Protein yield was 2–4% of original cell protein, containing 23–34% of succinate dehydrogenase activity measured in whole cells.

Microsomal preprations. For isolating microsomes, the resultant supernate of the first high-spin mitochondria centrifugation was again centrifuged at 96000 *g* for 30 min. The resulting microsomal pellet was washed once in a modified medium A containing 1 mM mercaptoethanol instead of EGTA and serum albumin. No contaminating mitochondria were detected by electron microscopy. Protein yield was 8–11% of total cellular protein. No succinate dehydrogenase activity could be detected. Approximately 40% of total glucose-6-phosphatase activity was associated with this fraction ($n = 2$).

[$^{45}Ca^{2+}$] uptake by isolated mitochondria and microsomes. Washed mitochondria were resuspended in ice cold medium B containing (final concentrations) 100 mM mannitol, 25 mM sucrose, 50 mM KCl, 1 mM $MgCl_2$, 2 mM KH_2PO_4, 0.5% fatty acid free bovine serum albumin, 10 mM HEPES, adjusted to pH 7.2. After 2–3 min a respiratory substrate solution was added to the mitochondrial suspension (final concentrations: 5 mM malate, 5 mM glutamate, 10 mM succinate, 10 mM ATP, pH 7.2). Radioactive [$^{45}Ca^{2+}$] (30 mCi/mg, New England Nuclear) was then added (150 μCi; final Ca^{2+} concentration, 0.2 mM) and the mixture was incubated at 37°C. Samples were drawn at the times indicated in the figures and mixed with ice cold medium B supplemented with 5 mM EGTA and 8 μM ruthenium red (1:5 v/v). This quench medium was used to inhibit Ca^{2+} fluxes (24). Mitochondria were then separated by centrifugation (12,000 *g*; 2 min) and the supernate was removed by aspiration. The resulting pellet was washed once with ice cold quench medium in which serum albumin had been omitted. It was then dissolved in 0.1% Triton X-100. Radioactivity was monitored using the liquid scintillation technique, protein content was determined by the Lowry method (21). To determinate contaminating [$^{45}Ca^{2+}$], mitochondria were incubated as mentioned in the absence of radioactive Ca^{2+}. The quence medium was then added, followed by radioactive [$^{45}Ca^{2+}$]. The amount of radioactivity incorporated in these reactions was generally less than 9% of the maximal [$^{45}Ca^{2+}$] uptake and subsequently subtracted. If inhibitors were used in mitochondria experiments, the controls were done with the same amount of solvent used to dissolve the inhibitor.

Experiments with microsomes were performed similar to those with mitochondria. After incubation and addition of the quench medium the resulting mixture was not centrifuged, but filtered and washed with quench medium on a 0.45-μ millipore filter. Microsomal protein was determined before resuspension in medium B in a small aliquot as described above. [$^{45}Ca^{2+}$] adhering to the filter was measured by liquid scintillation techniques.

Preparation of submitochondrial particles. For measurement of electron transfer activities, submitochondrial particles were isolated by sonifying isolated mitochondria with a Branson Sonifier, Modell S 75, in potassium phosphate buffer (100 mM, adjusted to pH 7.5) at 0–4°C. The homogenate was then first centrifuged at 8000 *g* for 5 min to remove larger particles and still intact mitochondria. Subsequently centrifugation was performed at 144,000 *g* for 60 min to yield a glassy, greenish-brown pellet (submitochondrial particles). Recovery was approximately 40–60% of total mitochondrial protein and 60–85% of succinate dehydrogenase activity.

Enzymatic assays. Spectrophotometric measurements were carried out using a Cary 219 spectrophotometer equipped with waterjacketed cell holders, which were controlled to ± 0.5°C of the desired temperature. Enzyme activities are expressed as $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$. *NADH dehydrogenase* was measured using ferricyanide as electron acceptor according to the method described by Galante and Hatefi (13). The reaction was monitored at 410 nm and was not inhibited by rotenone. *Succinate dehydrogenase* was determined by the method of Ackrell *et al.* (1), using the artificial electron acceptor phenazine methosulfate and cytochrome c as terminal oxidant. Antimycin A was added to assure that no electron flux to cytochrome c and O_2 occurs via the respiratory chain. *NADH cytochrome c reductase* determinations were carried out according to Hatefi and Stignall (15), except for the omission of phospholipid suspensions. Rotenone insensitive contributions were usually less than 14% and subsequently substracted in all experiments. *Succinate cytochrome c reductase* measurements were carried out as described by King (19). *Cytochrome c oxidase* is conveniently assayed by the spectrophotometric method of Smith as described by Errede *et al.* (10). A buffer containing 0.1 M 2-(N-morpholino)ethanesulfonic acid was used. For ferrocytochrome c preparations cytochrome c from Sigma was used. The decrease of absorbance of ferrocytochrome c was observed at 550 nm. The reaction was blocked by sodium azide. *NADH-oxidase* was determined polarographically and spectrophotometrically. Polarographic activities were measured using a Clark-type electrode (Yellow Springs Instrument Co.). A modified method described by King (20) was applied. The assay medium contained 100 mM KCl, 20 mM HEPES, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.5 mM EDTA, 0.1%

fatty acid free bovine serum albumin, pH 7.2. Cytochrome c (3 μM) and carbonylcyanide m-chlorophenylhydrazone (5 μM) were added shortly before measurements were started. To get appreciable tracings with the oxygen electrode, relatively large amounts of submitochondrial protein were necessary, therefore, a new spectophotometric method was developed as suggested by von Jagow (17). This method measures the decrease of NADH in the presence of submitochondrial particles. The assay medium contained 250 mM sucrose, 50 mM potassium phosphate, 5 mM $MgCl_2$, 0.2% fatty acid free bovine serum albumin, 0.2 mM EDTA, and 2 μM carbonylcyanide m-chlorophenylhydrazone, adjusted to pH 7.3. Submitochondrial particles were added and the reaction was started with NADH. Monitoring occurred at 340 (−420) nm. Addition of antimycin A inhibited the NADH oxidation completely. Sodium azide inhibited the reaction after an initial decrease of NADH, probably due to ferricytochrome c in submitochondrial preparations. *Statistical analysis.* the Student's *t* test was used.

Materials. If not stated otherwise all biochemical reagents were obtained from Sigma Chemicals, St. Louis—Munich, Federal Republic of Germany. Salts and solvents were of the purest grade from E. Merck, Darmstadt, Federal Republic of Germany.

RESULTS

Intracellular Ca^{2+} in CF, obligate heterozygote, and control fibroblasts. Figure 1 demonstrates that intracellular Ca^{2+} increased with culture passage (weekly age) in CF and control cells. Average Ca^{2+} in CF fibroblasts increased from 9.0 (passage 2) to 10.6 (passage 15) nmol/mg cellular protein. Ca^{2+} in control cells increased from 5.1 (passage 2) to 7.8 (passage 16) nmol/mg cellular protein. No regression line was plotted for CF heterozygote cells because sufficient data was not present. Sex, age, and clinical status of the fibroblast donors studied were not rated separately because the differences in intracellular Ca^{2+} were not significant. In the case of CF cells, the standard deviation varied between 13–18% of the mean intracellular Ca^{2+} value at different passages (controls, 12–16%). Statistical analysis showed that Ca^{2+} was significantly elevated in CF cells at all culture passages, if compared with controls or heterozygote cells ($P < 0.03$). But as Figure 1 shows, intracellular Ca^{2+} is not a reliable criterion for cell discrimination, because of the high variability of results.

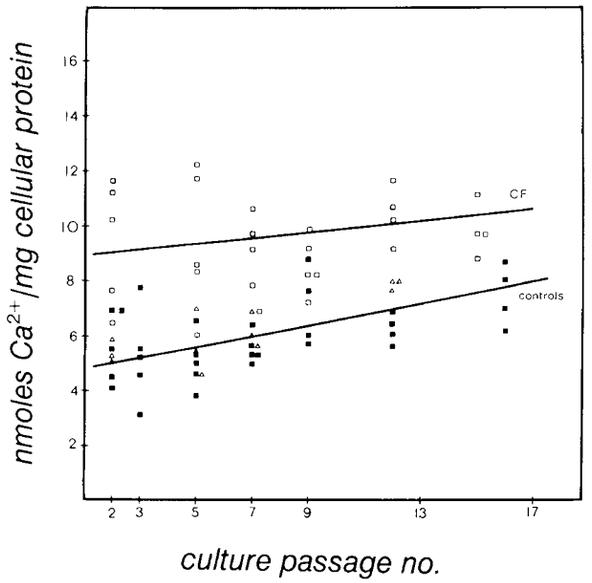

Fig. 1. Intracellular calcium in fibroblasts from patients with cystic fibrosis (CF) (□), obligate heterozygotes (△), and controls (■) as determined by isotope detection. Each symbol represents the mean of duplicate determinations of five different cultures from five different patients (see "Materials and Methods"). Ages of CF patients ranged from 14–25 yr ($n = 25$); obligate heterozygotes, 21–33 yr ($n = 14$); and controls, 14–30 yr ($n = 23$). The regression lines represent average intracellular Ca^{2+}, presuming that Ca^{2+} content increases at a constant rate with cell culture age (culture passage). No regression line was plotted for obligate heterozygote fibroblasts.

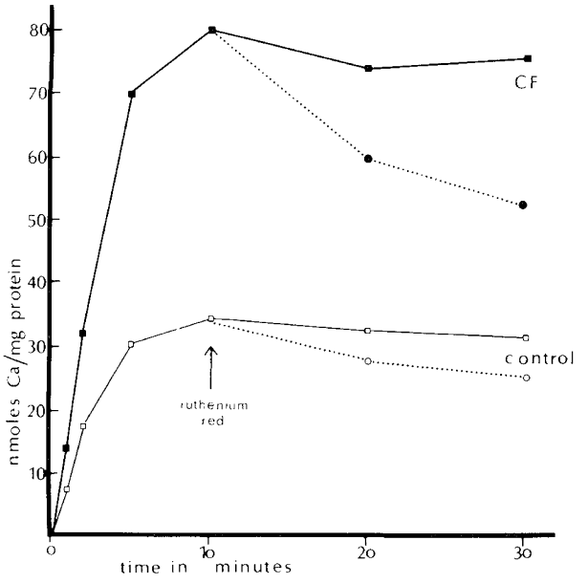

Fig. 2. Kinetic analysis of calcium uptake by mitochondria isolated from cystic fibrosis (CF) fibroblasts (■, ●) and controls (□, ○). Each symbol represents the mean of duplicate and simultaneous determinations of Ca^{2+} uptake in mitochondria from one CF and one control cell strain (cell passage 8). After 10 min the mitochondrial Ca^{2+} influx inhibitor ruthenium red (final concentration 2×10^{-5} mol/l) was added to part of the incubation medium and uptake studies continued with (●, ○) and without (■, □) ruthenium red. Mitochondrial protein was less than 0.5 mg/ml. Ca^{2+} uptake was calculated as described (see "Materials and Methods"). The picture shown is representative for $n = 11$ studies (see Table 1).

Ca^{2+} uptake by isolated cell organelles (mitochondria, microsomes). In vitro uptake of Ca^{2+} in mitochondria and microsomes isolated from CF and control fibroblasts is demonstrated in Figure 2 and Table 1. Figure 2 shows the time course of Ca^{2+} uptake by mitochondria. Apparently no further uptake of Ca^{2+} occured in CF mitochondria and controls after a 10-min incubation period. The bulk of Ca^{2+} had entered the mitochondria by 5 min. Under test conditions, maximal Ca^{2+} uptake was estimated to be in the range of 50–100 nmol·mg mitochondrial protein^{-1}·min^{-1} for CF mitochondria (controls, approximately 30–70 nmol Ca^{2+}·mg^{-1}·min^{-1}). Table 1 shows that CF mitochondria can accumulate about 2.4 times more Ca^{2+} than controls and about 2.1 times more than obligate heterozygote mitochondria.

Figure 2 also demonstrates that ruthenium red, an inhibitor of mitochondrial Ca^{2+} influx, reduced mitochondrial Ca^{2+} pools if added to the incubation medium after Ca^{2+} loading had occurred (10 min after start). The maximal Ca^{2+} efflux in the case of CF mitochondria was estimated to be 2–3 nmol·mg mitochondrial protein^{-1}·min^{-1} and was 3–4 times larger than in controls. Because no net uptake of Ca^{2+} occurred in the absence of ruthenium red after incubating mitochondria for 10 min, it must be assumed that an equilibrium of efflux and influx takes place after the initial Ca^{2+} uptake phase in both mitochondria from CF and controls.

Table 1 shows also that unspecific (contaminating) Ca^{2+} in mitochondria did not exceed 12% of maximal Ca^{2+} uptake. This

was measured by blocking energy-dependent mitochondrial Ca^{2+} influx by ruthenium red and inhibitors of respiration and ATP hydrolysis. Statistical analysis of the differences in Ca^{2+} uptake of CF mitochondria and controls or heterozygote mitochondria were highly significant ($P < 0.0005$). Mitochondrial Ca^{2+} uptake never failed to distinguish homozygous CF cell strains in our tests ($n = 11$).

A comparison between microsomes from CF and control fibroblasts (Table 1) showed that there were no significant differences in Ca^{2+} accumulation either in the presence or absence of mitochondrial Ca^{2+}-uptake inhibitors ($P > 0.025$). Because of the large amounts of cells needed to isolate microsomes and mitochondria (approximately $5–10 \times 10^7$), culture passage 8 was uniformly studied in these tests. The mitochondrial or microsomal Ca^{2+} uptake was not rated according to sex, age, or clinical status of cell donor because these differences were not observed as significant within the tested groups.

Inhibition of energy-dependent mitochondrial Ca^{2+} influx. To localize possible abnormalities of mitochondrial energy metabolism responsible for mitochondrial Ca^{2+} uptake, different reagents were used to inhibit energy processing at different sites. A quantitative analysis of the influence of these inhibitors on mitochondrial Ca^{2+} uptake is shown in Table 2. Only 0 time and 10-min assays were carried out in these tests, contrary to the kinetic analysis mentioned in Table 1 and Figure 2.

Table 1. *Calcium uptake by mitochondria and microsomes isolated from fibroblasts of patients with cystic fibrosis (CF), obligate heterozygotes, and controls**

	Ca^{2+} uptake [nmol $Ca^{2+} \cdot$ mg protein$^{-1} \cdot 10$ min^{-1} (±SD)]†		
	CF	Heterozygotes	Controls
Mitochondria	79.5 ± 8.2	39.2 ± 4.3	33.7 ± 4.7
Microsomes	13.4 ± 1.4	11.1 ± 1.2	9.8 ± 0.9
Mitochondria (+RASO)‡	3.7 ± 0.9	ND	2.8 ± 1.2
Microsomes (+RASO)‡	11.2 ± 0.8	ND	9.1 ± 0.3

* Each value represents the mean of duplicate determinations of 11 (mitochondria) or five (microsomes) cell strains. Experiments with RASO were done with two cell strains for each mitochondria and microsomes. Mitochondrial Ca^{2+} uptake values were derived from kinetic analysis as shown in Figure 2 (see "Methods and Materials"). ND, none detected.

† Ca^{2+} uptake after the first 10 min in vitro incubation period was rated.

‡ RASO, 20 μM ruthenium red, 10 μM antimycin A, 2mM sodium azide, and 5 μM oligomycin (final concentrations).

Table 2 demonstrates the following points concerning inhibitors. *Rotenone* (inhibits complex I of the respiratory chain) did not significantly influence Ca^{2+} uptake in CF or control mitochondria; however, on an average, it did reduce Ca^{2+} uptake more in CF mitochondria. *Antimycin A* (inhibits electron transport in respiratory chain at complex III) decreased Ca^{2+} uptake by an average of 38% in CF mitochondria, and 26% in controls. Again, these differences were not significant, but CF mitochondria did seem influenced more by antimycin A. *Oligomycin* (blocks ATP synthase or hydrolysis and has an inhibitory effect on electron transport in respiration) decreased Ca^{2+} uptake in CF mitochondria by an average of 61%, but by only 46% in controls. This difference in inhibitory action was significant ($P < 0.05$). *Atractyloside* (limits and inhibits adenine nucleotide transport across the mitochondrial membrane) lowered Ca^{2+} uptake in CF mitochondria by an average of 39%, but only 12% in controls. This difference was also significant ($P < 0.05$). The combined action of ruthenium red, antimycin A, and oligomycin decreased mitochondrial Ca^{2+} uptake by 92–96% in CF mitochondria and 84–88% in controls. Contamination rates were comparable to this remaining Ca^{2+} uptake (see "Materials and Methods").

Electron transfer activities in submitochondrial particles. The experiments with inhibitors of mitochondrial energy metabolism could not eliminate the possibility that electron transport in the respiratory chain may also be affected in CF mitochondria. Figure 3 shows the relative electron transfer activities in CF, obligate heterozygote, and control submitochondrial particles. The action of single redox components or complex entities of the respiratory chain like NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), and cytochrome c oxidase (complex IV) was not influenced in all cell strains (Fig. 3 a,b,c). On the other hand, the electron transfer activities markedly changed if two or more redox components of the respiratory chain were involved like in NADH oxidase (complexes I, III and IV), NADH cytochrome c reductase (complexes I and III), and succinate cytochrome c reductase (complexes II and III). This can be observed in Figure 3 d,e,f. Comparing CF mitochondrial particles with controls, the average corresponding increases in transfer activities were 77, 58, and 48%, respectively. In the case of obligate heterozygote *versus* control mitochondria increases were 14, 15, and 22%, respectively. These increases of electron transfer activities in submitochondrial particles of CF and obligate heterozygote CF cells were all significant ($P < 0.05–0.001$).

DISCUSSION

Experiments with fibroblasts grown under optimal conditions have the advantage that the findings are probably not secondarily the consequence of chronic disease or malabsorption as for instance in material derived directly from patients with CF.

Table 2. *Effect of different inhibitors on calcium uptake of mitochondria isolated from cystic fibrosis (CF) and control fibroblasts**

		Ca^{2+} uptake	
		CT mitochondria	Controls
		[nmol $Ca^{2+} \cdot$ mg protein$^{-1} \cdot 10$ min^{-1} (±SD)]	
No inhibitor		83.8 ± 9.4 (100)†	36.7 ± 5.1 (100)†
Inhibitor	(mol/l)		
rotenone	10^{-5}	70.4 ± 8.4 (84)	34.1 ± 4.4 (93)
antimycin A	10^{-5}	51.9 ± 5.9 (62)	27.1 ± 4.0 (74)
oligomycin	0.5×10^{-5}	32.7 ± 4.1 (39)	19.8 ± 2.6 (54)
atractyloside	10^{-5}	51.1 ± 4.2 (61)	32.3 ± 3.3 (88)
ruthenium red	10^{-5}		
+ antimycin A	10^{-5}	5.0 ± 1.7 (6)	5.1 ± 0.8 (14)
+ oligomycin	0.5×10^{-5}		

* Each value represents the mean of duplicate determinations of seven cell strains. Ca^{2+} associated with mitochondria after 0 min incubation was subtracted from Ca^{2+} uptake after 10 min *in vitro* incubation. No kinetic analysis were performed as shown in Figure 2. Ca^{2+} uptake was promoted by malate, glutamate, succinate and ATP as described in "Materials and Methods."

† Number in brackets indicates relative Ca^{2+} uptake, in %.

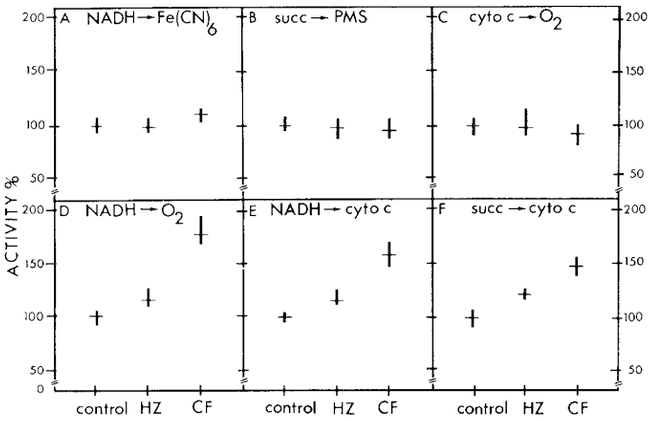

Fig. 3. Relative electron transfer activities in submitochondrial particles from fibroblasts of patients with cystic fibrosis (CF), obligate heterozygotes (HZ), and controls. Each *vertical bar* represents the range of relative electron transfer activities and the *horizontal line* indicates the mean value. The control mean value was arbitrarily set 100%. Duplicate determinations of electron transfer activities of at least seven different cell strains (cell passage 8) were rated. The ages of cell donors were as described in Figure 1. For enzymatic assays see "Materials and Methods." (*A*) NADH-dehydrogenase, (*B*) succinate-dehydrogenase, (*C*) cytochrome c oxidase, (*D*) NADH-oxidase, (*E*) NADH-cytochrome c reductase, and (*F*) succinate-cytochrome c reductase.

Parameters like age, sex, Shwachmann scores, or case history of cell donors were not observed as significant in our tests and cell cultures. The results show that intracellular Ca^{2+} content is elevated by 36–77% in CF fibroblasts and depends on the age (passage) of cell culture. Recently similar findings were reported by Shapiro and Lam (29). In a previous study, we were able to confirm that intracellular Ca^{2+} is also significantly elevated in CF leukocytes (2, 25, 28). The discrimination of different cell strains (CF, obligate heterozygote, control) by intracellular Ca^{2+} content was not reliable because of the high variability of results, not uncommon in tissue culture or leukocyte experiments. Nevertheless, high Ca^{2+} levels in cells may help identify CF in special cases (*e.g.*, small infants) because intracellular Ca^{2+} determination by isotope detection is easily and cheaply done with small amounts of cells.

The cell cannot store high amounts of Ca^{2+} in the cytosol without severe damage or even death (4, 6, 12). It seemed, therefore, reasonable to expect Ca^{2+} accumulation in either mitochondria or the endoplasmic reticulum of CF cells. Together these cell organelles store 95–98% of intracellular Ca^{2+} (6, 12). Our experiments showed that isolated CF mitochondria take up 2–3 times as much Ca^{2+} as controls or heterozygote CF mitochondria. Maximal Ca^{2+} influx and efflux rates were of the same magnitude as those reported for rat liver mitochondria by other authors (12) (V_{max} = 50–100 and 2–3 nmol $Ca^{2+} \cdot$ mg mitochondrial protein$^{-1} \cdot$ min^{-1}, respectively). On the other hand, the endoplasmic reticulum (microsomes) of CF cells did not accumulate more Ca^{2+} in our experiments. Mitochondrial Ca^{2+} uptake accurately distinguished between CF cell strains and controls or CF heterozygotes (Table 1). For clinical trials, *i.e.*, prenatal diagnosis of CF, Ca^{2+} uptake in mitochondria does not seem practical unless a cheap microassay needing only a small amount of cells can be developed. Elevated uptake of Ca^{2+} in CF mitochondria was first observed by Feigal and Shapiro (11).

It has been pointed out in the literature that increased intracellular Ca^{2+} may be substantial in CF (30, 32). In the cell, the most important action site of Ca^{2+} is the cytosol, where it performs as a messenger (5, 12, 23); therefore, it is essential if increased intracellular Ca^{2+} in CF cells means only higher intramitochondrial or also changed cytosolic Ca^{2+} levels. Our investigations have shown that Ca^{2+} uptake *and* Ca^{2+} efflux are elevated differently in isolated CF mitochondria. This suggests that the steady-state of cytosolic Ca^{2+} can be affected in CF cells.

In intact mitochondria Ca^{2+} efflux is regulated by an antiport Ca^{2+} translocase that is different from the energy-dependent uniport Ca^{2+} translocase responsible for Ca^{2+} uptake (6). If a higher efflux of Ca^{2+} occurs in CF mitochondria after loading with radioactive Ca^{2+}, this may be due to environmental or intrinsic changes of the antiport Ca^{2+} translocase or to increased levels of Ca^{2+} in CF mitochondria (6, 22). On the basis of our experiments neither possibility can be ruled out.

Uptake of Ca^{2+} by mitochondria is energy-dependent. Our experiments have shown that there are significantly different effects on Ca^{2+} uptake in CF and control mitochondria by ATP transport inhibitors (atractyloside) and ATP hydrolysis inhibitors (oligomycin) (Table 2). Furthermore, the electron transfer activities of various enzymes or redox components of the respiratory chain (NADH oxidase, NADH cytochrome c reductase and succinate cytochrome c reductase) were significantly changed in CF mitochondria. These multiple changes of energy processing all involve functions that are associated with the inner mitochondrial membrane. Because CF is presumably a monogenic disease and a single gene must account for these different changes, it seems reasonable that one essential modification of the mitochondrial inner membrane has occurred in CF, changing the action of many membrane-associated enzymes and functions. Nevertheless specific intrinsic changes of the energy-processing enzymes mentioned cannot be ruled out (30).

The finding that electron transport in the respiratory chain is only influenced if two or more redox components are involved (Fig. 3) gives a clue as to what kind of modification may be present in CF mitochondria. Recently, the significance of phospholipids for eliciting the specific catalytic reactions along the electron transfer sequence was demonstrated (14). Increases of electron transfer activities correspond to decreases of phospholipid content in the mitochondrial inner membrane and vice versa. The intrinsic capacity of single redox components such as NADH dehydrogenase and cytochrome c oxidase was not influenced by phospholipid content, but electron transfer was much influenced by phospholipids if two or more redox components were involved (14, 26, 27). This correlates well with our results and suggests that a defect of membrane phospholipid metabolism may be present in CF mitochondria.

Significant increases of electron transfer activities in coupled redox components were also observed in submitochondrial particles from CF heterozygote cells (Fig. 3 d,e,f). Compared with CF mitochondrial particles, these increases were small but they correlate with the somewhat elevated Ca^{2+} uptake found in heterozygote mitochondria compared with controls (Table 1). Apparently this mild derangement of mitochondrial energy processing does not greatly effect mitochondrial and cellular Ca^{2+} uptake (Table 1 and Fig. 1). In summary our results suggest that the disorder of Ca^{2+} metabolism observed in CF cells probably is generated by a defect of energy *and* membrane metabolism in CF mitochondria.

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Pre- and Postnatal Development of Granulocytic Stem Cells in the Rat

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Summary

Bacterial sepsis is a relatively common problem in the neonatal period, particularly among prematurely delivered infants. The newborn rat has been widely used as a model for sepsis neonatorum, and in that model incomplete development of the neutrophil system has been postulated to be an important factor predisposing neonates to death from bacterial infection. In this study, that hypothesis was further tested by assessing neutrophil development in rats of various pre- and postnatal ages. Using standard soft agar colony techniques for detecting granulocyte-macrophage progenitor cells [CFU(c)], the number of CFU(c)/g of body weight was seen to increase from $0.5 + 0.1 \times 10^3$ at 19–20 days gestation to $10.5 \pm 0.2 \times 10^3$ at 4 weeks. The anatomic location of CFU(c) changed from totally hepatic at 16 days gestation to almost totally myeloid at 4 weeks. Lastly, the proportion of mature, stored neutrophils/CFU(c) decreased from 2440 ± 40 at 19–20 days gestation to 430 ± 75 at 4 weeks.

Abbreviations

CFU(c), colony-forming unit in culture
PMN, polymorphonuclear leukocyte
M5A, McCoy's 5A medium

Very early in gestation, it is unlikely that a fetus, existing in a sterile environment, requires the antimicrobial neutrophil system. Indeed, although red blood cells circulate in the human fetus before the 5th week of gestation (19), mature neutrophils generally do not appear until after the 12th to 14th week (13) and then exist only in very minute concentration until after birth (16). Because of this late appearance of neutrophils during human gestation, we postulated that infants born prematurely might possess an incompletely developed neutrophil system and that this deficiency might, in part, be responsible for the high incidence of bacterial infection in this group of patients (18). In