

Mechanisms for Intracellular Calcium Regulation in Heart

I. Stopped-Flow Measurements of Ca⁺⁺ Uptake by Cardiac Mitochondria

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ABSTRACT Initial velocities of energy-dependent Ca⁺⁺ uptake were measured by stopped-flow and dual-wavelength techniques in mitochondria isolated from hearts of rats, guinea pigs, squirrels, pigeons, and frogs. The rate of Ca⁺⁺ uptake by rat heart mitochondria was 0.05 nmol/mg/s at 5 μ M Ca⁺⁺ and increased sigmoidally to 8 nmol/mg/s at 200 μ M Ca⁺⁺. A Hill plot of the data yields a straight line with slope n of 2, indicating a cooperativity for Ca⁺⁺ transport in cardiac mitochondria. Comparable rates of Ca⁺⁺ uptake and sigmoidal plots were obtained with mitochondria from other mammalian hearts. On the other hand, the rates of Ca⁺⁺ uptake by frog heart mitochondria were higher at any Ca⁺⁺ concentrations. The half-maximal rate of Ca⁺⁺ transport was observed at 30, 60, 72, 87, 92 μ M Ca⁺⁺ for cardiac mitochondria from frog, squirrel, pigeon, guinea pig, and rat, respectively. The sigmoidicity and the high apparent K_m render mitochondrial Ca⁺⁺ uptake slow below 10 μ M. At these concentrations the rate of Ca⁺⁺ uptake by cardiac mitochondria in vitro and the amount of mitochondria present in the heart are not consistent with the amount of Ca⁺⁺ to be sequestered in vivo during heart relaxation. Therefore, it appears that, at least in mammalian hearts, the energy-linked transport of Ca⁺⁺ by mitochondria is inadequate for regulating the beat-to-beat Ca⁺⁺ cycle. The results obtained and the proposed cooperativity for mitochondrial Ca⁺⁺ uptake are discussed in terms of physiological regulation of intracellular Ca⁺⁺ homeostasis in cardiac cells.

INTRODUCTION

It is well accepted that in skeletal muscle, the sarcoplasmic reticulum controls the state of contraction and relaxation of the myofibrils by regulating the intracellular calcium ion concentrations (1-5). Although the involvement of calcium in the contractile cycle of skeletal and cardiac muscle is

similar (6–8), the role of sarcoplasmic reticulum in heart muscle is less clear and has been questioned by a number of investigators (9–13). The rationale of this questioning rests on the fact that the sarcoplasmic reticulum is poorly represented in heart (14, 15) and shows reduced ability to accumulate Ca^{++} in vitro (1, 3, 5, 16). In cardiac cells, however, mitochondria are very abundant, are located in regular rows in proximity of the myofibrils (15), and have a high Ca^{++} -transport activity. Consequently, the mitochondria have been proposed to play an important role in the regulation of excitation contraction coupling of heart, by taking up and releasing significant amounts of Ca^{++} during the contractile cycle. Although during the last 10 years numerous reports have implicated the mitochondria as the relaxing factor in heart (9, 10, 12–13, 17), most of these studies have been criticized on the grounds of the limitations of the technique used and misinterpretation of the data obtained (3, 7, 18–20). Presently, the evidence for a major role of mitochondria in the regulation of the beat-to-beat Ca^{++} cycle in heart appears inconclusive (3, 7).

In this study, energy-linked Ca^{++} uptake was measured in mitochondria isolated from the hearts of various animal species using stopped-flow and dual-wavelength techniques. The purpose of the investigation was to ascertain the amount of mitochondria present in the myocardium of different species and the amount of Ca^{++} taken up in vitro by mitochondria during the time described for heart relaxation, and to determine whether this was consistent with the calculated amount of Ca^{++} sequestered in vivo during relaxation. In addition, initial velocities of Ca^{++} uptake were measured in isolated cardiac mitochondria at Ca^{++} concentrations similar to those reported for cytosolic Ca^{++} levels (1–3, 8, 21), and the apparent affinity of the Ca^{++} -transport system in mitochondria from various hearts was calculated. The results obtained indicated that, at least in mammalian hearts, the energy-linked Ca^{++} uptake of mitochondria is inadequate for the regulation of the beat-to-beat Ca^{++} cycle. A preliminary report of this work has been presented (22).

MATERIALS AND METHODS

Isolation of Mitochondria

Sprague-Dawley male rats (200–300 g), male Hartley strain guinea pigs (500–700 g), male pigeons (*Columba livia*, 150–250 g), ground squirrels (100–150 g), and frogs (*Rana pipiens* or *Rana catesbeiana*, 50–300 g) were used as experimental animals. The animals were sacrificed by decapitation and their hearts were removed quickly and perfused with 0.25 M sucrose. The left and right ventricula were trimmed of fat and connective tissue, finely chopped, and washed several times in ice-cold solution containing 0.225 M mannitol, 0.075 M sucrose, and 0.5 mM EDTA, pH 7.4 (MSE medium). The minced tissue was then diluted with MSE medium (10 ml/g tissue)

and homogenized with a Polytron tissue disintegrator, model PT10 (Brinkmann Instruments, Inc., Westbury, N. Y.) for 15 s with the rheostat set in position 4. The homogenate was diluted with 4 vol of MSE medium and centrifuged at 9,000 *g* for 15 min. The resulting mitochondrial pellet was separated from the fluffy layer and resuspended with a solution containing 0.225 M mannitol and 0.075 M sucrose. This procedure was repeated twice and the final mitochondrial pellet was resuspended using a small glass homogenizer with the sucrose-mannitol medium to yield a uniform suspension containing 50–70 mg protein/ml. All operations were carried out at 4°C. Protein concentration was determined by the biuret method.

Content, Yield, and Properties of Mitochondria

The amount of mitochondria present in the tissue *in vivo* was estimated by measuring and comparing the cytochrome *a* + *a*₃ content and cytochrome *c* oxidase activities in both the isolated mitochondria and initial homogenate. A suitable homogenate was prepared by finely dispersing 0.5 g of minced heart tissue in 40 ml of 20 mM K₂HPO₄ (pH 7.2) with the aid of the Polytron disintegrator (2 min at half-maximal speed). Cytochrome *a* + *a*₃ content of homogenates or mitochondria was measured by dual-wavelength spectroscopy (23) at 605–620 nm as described by Williams (24), in media containing 100 mM K₂HPO₄ (pH 7.2). Cytochrome *c* oxidase was measured as described by Smith and Conrad (25) and Yonetani (26) at 550–540 nm by recording the absorbance decrease of ferrocytochrome *c* in reaction media containing 100 mM K₂HPO₄ (pH 7.2), 100 μM ferrocytochrome *c*, and aliquots of mitochondria and homogenate. Cytochrome *c* was reduced by Na₂S₂O₄ and the dithionite was removed by overnight dialysis under nitrogen against 1 mM phosphate (pH 7.2). Calculations use the extinction coefficients for cytochrome *a* + *a*₃ and *c* reported by van Gelder and Slater (27).

Respiratory control ratios were measured by adding 0.1 ml of mitochondrial preparation (about 5 mg protein) to 2.4 ml of a medium containing 0.25 M sucrose, 15 mM Na morpholinopropane sulphonate (MOPS) (pH 7.4), 10 mM K₂HPO₄. Sodium malate and sodium glutamate (8 mM each) were added to initiate state 4 respiration (28) and then 280 μM ADP was added to induce state 3. Oxygen consumption was measured polarographically with a Clark-type electrode in a thermostated reaction vessel equipped with a magnetic stirrer, and monitored with a potentiometric recorder. Respiratory control ratios were calculated according to Chance (28) as the ratio of the rate of respiration in the presence of added ADP and the rate obtained after ADP expenditure.

Ca⁺⁺ Measurements

Kinetic measurements of Ca⁺⁺ movement were measured spectrophotometrically in reaction mixtures containing 0.15 M sucrose, 0.075 M KCl, 2 mM K₂HPO₄ or potassium acetate, 3 mM MOPS pH 7.2, 5 mM MgCl₂, 1.7 mM sodium succinate, 3 μM rotenone, 35 μM murexide, and 1–7 mg mitochondrial protein/ml. In some experiments, sodium succinate and rotenone were substituted by 2 mM sodium malate and 2 mM sodium glutamate. The temperature of the reaction medium was 26°C. The Ca-murexide complex, as compared with murexide alone, exhibits a lower

absorbance at 540 nm and a higher absorbance at 470-nm wavelength, with an isobestic point at 507 nm (29–31). In the presence of murexide, Ca⁺⁺ uptake by mitochondria produces an increase in absorbance at 540 nm by reducing the concentration of Ca⁺⁺ available for the formation of the calcium-murexide complex. In order to minimize nonspecific absorbance changes, the experiments reported were carried out with a dual-wavelength spectrophotometer (32) which subtracted the changes in absorbance at 507 nm (reference wavelength) from the changes at 540 nm (measured wavelength).

Fast mixing of Ca⁺⁺ to the reaction mixture was obtained with the aid of a stopped-flow apparatus designed by Chance (33) and built in our department. The device mixes reactants with a ratio of 1:160 in less than 1 ms (34). Absorbance changes and flow velocity traces were displayed in a storage oscilloscope as described previously (31, 35–36).

Materials

Carbonyl cyanide-*p*-trifluoromethoxy phenylhydrazone (FCCP) was a gift from Dr. P. G. Heytler of E. I. Du Pont de Nemours & Co., Wilmington, Del. Murexide was purchased from K & K Laboratories, Inc., Plainview, N. Y., antimycin A from Calbiochem, San Diego, Calif., cytochrome *c* type III, sodium adenosine-5'-diphosphate (ADP), grade 1 and morpholinopropane sulphonic acid (MOPS) from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Table I shows estimates of the amount of mitochondria present in vivo in 1 g wet wt of heart tissue from rat, pigeon, and frog, together with the yield and respiratory control ratio after isolation. The estimated amount of mitochondria present in 1 g cardiac tissue is three times higher than that reported

TABLE I
ESTIMATION OF AMOUNT OF MITOCHONDRIA IN HEARTS FROM RAT,
PIGEON, AND FROG AND THE YIELD AND RESPIRATORY CONTROL
RATIO AFTER THEIR ISOLATION

Mitochondrial content was estimated by measuring and comparing the content of cytochrome oxidase (in *a*) or the activity of ferrocytochrome *c* oxidase (in *b*) in isolated mitochondria and in tissue homogenate, as described in Materials and Methods. The values reported represent the average of three different experiments. Values of the yield of mitochondria indicate the variability range of four different preparations. Respiratory control ratio (RCR) was measured as described in Methods in the presence of 1.4 mg/ml of mitochondrial protein and 8 mM sodium glutamate and 8 mM sodium malate.

Tissue	Mitochondrial content in the tissue		Yield	RCR
	<i>a</i>	<i>b</i>		
	<i>mg mitochondria/g wt tissue</i>		<i>mg mitochondria/g tissue</i>	
Rat heart	81±7	73±11	4.3–7.1	6.5–9.3
Pigeon heart	92±19	84±16	3.9–6	5.5–8.1
Frog heart	77±6	71±12	3.1–5.8	4.5–7.3

by Slater and Cleland (37) and only 30% higher than that of Klingenberg et al. (38). Slightly higher values were obtained by calculating the amount of mitochondria present in vivo on the basis of cytochrome $a + a_2$ content and this may be accounted for by small myoglobin interference at the wavelength used. The yield of mitochondria was lower than in other reports (1, 3, 37, 38) and this may be attributed to the systematic removal of the light mitochondria from the pellet during washing procedures. Although values from preparation of frog heart mitochondria are not available in the literature, Table I shows that these mitochondria can be isolated with yields and respiratory control ratios comparable to those of other cardiac mitochondria.

The initial velocities of Ca^{++} uptake by heart mitochondria were measured by recording the absorbance changes of the Ca^{++} indicator, murexide, by dual-wavelength spectroscopy after the rapid mixing of Ca^{++} into the reaction mixture with a stopped-flow apparatus. Fig. 1 illustrates the system used for these experiments. The larger syringe of the flow apparatus contained the reaction mixtures together with murexide and mitochondria. The smaller

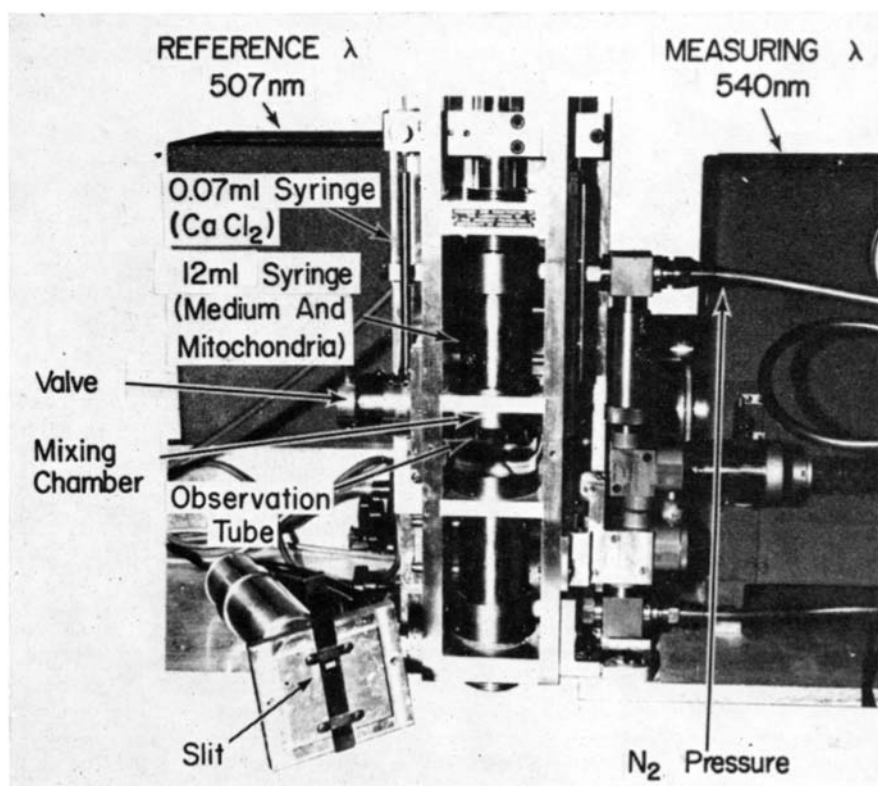


FIGURE 1. Dual-wavelength spectrophotometer and stopped-flow apparatus described in the text.

syringe contained CaCl_2 . The plungers of both syringes were pneumatically driven by high pressure nitrogen and CaCl_2 could be mixed with the reaction mixtures in less than 1 ms. The photomultiplier tube, located after the mixing chamber, observed the changes in absorbance of murexide at 540–507 nm from a few ms to several minutes after mixing. One of the important features of this stopped-flow apparatus is the presence of two syringes of unequal volume. This proves to be of particular importance in optical measurements in the presence of turbid solutions and makes possible addition of Ca^{++} to reaction mixtures with negligible dilution of the system as a whole.

Fig. 2 shows the recording traces of two experiments of Ca^{++} uptake obtained with a stopped-flow apparatus and dual-wavelength technique. The addition of 7.5 or 75 μM Ca^{++} to the reaction mixture through the stopped-flow apparatus produced an abrupt decrease in absorbance at 540 nm, due to the formation of the calcium murexide complex which absorbs less than murexide alone. The complexation of added Ca^{++} to murexide in the reaction medium was completed within the mixing time and was followed by a slow increase in absorbance related to the energy-dependent Ca^{++} uptake by mitochondria. Initial rates measured during the first 5 s were low; longer observation times (not shown) indicate that Ca^{++} uptake occurred at pro-

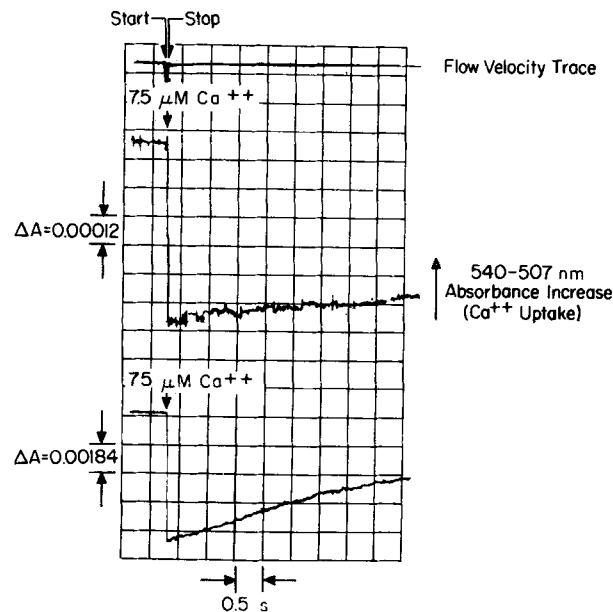


FIGURE 2. The kinetics of the energy-dependent Ca^{++} uptake in rat heart mitochondria. The large syringe of the stopped-flow apparatus contained 0.15 M sucrose, 0.075 M KCl, 2 mM K_2HPO_4 , 3 mM MOPS (pH 7.2), 5 mM MgCl_2 , 1.7 mM sodium succinate, 3 μM rotenone, 35 μM murexide, and 5.3 mg of mitochondrial protein/ml. 30 s after the addition of succinate the reaction was started by the discharge of either 7.5 or 75 μM CaCl_2 through the small syringe. Temperature, 26°C.

gressively slower rates, until all the Ca^{++} added was removed by the mitochondria.

As described above, the initial rates of the energy-dependent Ca^{++} uptake by rat heart mitochondria were measured after addition of various Ca^{++} concentrations. Fig. 3 shows that the initial velocity of Ca^{++} uptake was slow at low concentrations of Ca^{++} and increased sigmoidally to 10 nmol Ca^{++} /s/mg protein at 300 μM Ca^{++} . Similar results were obtained when mitochondria were oxidizing glutamate-malate, instead of succinate, when acetate was replacing phosphate as a permanent anion, and with a wide range of mitochondrial protein in the medium (0.5–10 mg/ml).

Although different preparations of rat heart mitochondria produced slight variations in the maximal velocity of Ca^{++} uptake (8–14 nmol/s/mg) and in the Ca^{++} concentration for half-maximal transport (85–105 μM), the plot of Ca^{++} uptake versus Ca^{++} concentrations resulted always in a sigmoidal curve. Fig. 4 shows that initial velocity of Ca^{++} uptake by rat heart mitochondria versus the added $[\text{Ca}^{++}]$ produced a nonlinear Lineweaver-Burk plot (Fig. 4 A). However, by substituting $[\text{Ca}^{++}]$ on the abscissa with the square of $[\text{Ca}^{++}]$, the same data produced a linear double reciprocal plot (Fig. 4 B). By plotting this data in the form of a Hill plot, a good straight line with a slope, n , of 2.06 was obtained (Fig. 4 C). All these data indicate the presence of a cooperative interaction in the energy-linked Ca^{++} uptake by rat heart mitochondria, requiring the existence of more than one binding site for Ca^{++} in the transport system.

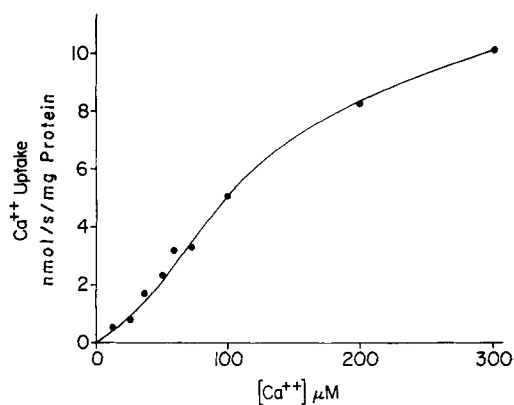


FIGURE 3. Initial rates of Ca^{++} uptake by rat heart mitochondria at various Ca^{++} concentrations. Experimental conditions similar to that of Fig. 2, except for the protein concentration of mitochondria which was 2.3 mg/ml. The reaction was initiated by discharging in the reaction mixtures the amount of Ca^{++} indicated in the abscissa. Initial velocity refers to the amount of Ca^{++} taken up by mitochondria during the first 200 ms after Ca^{++} additions. Every point was obtained with the same preparation of rat heart mitochondria within a few hours.

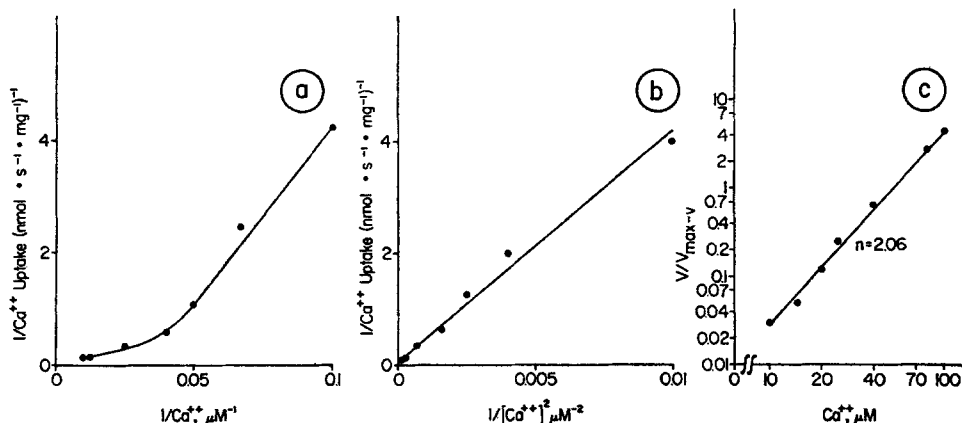


FIGURE 4. Double-reciprocal plots (a and b) and Hill plot (c) of Ca^{++} uptake by rat heart mitochondria. Experimental conditions as in Fig. 3, except for the protein concentration, which was 1.9 mg/ml.

The reported measurements of initial velocity of Ca^{++} uptake were carried out in reaction mixtures containing 5 mM MgCl_2 and 50 mM KCl . Both cations effectively compete with Ca^{++} for the low affinity, energy-independent Ca^{++} -binding sites in mitochondria (39) without interfering significantly with the energy-linked Ca^{++} transport (40). Fig. 5 shows that two additions of 4 μM Ca^{++} to reaction mixtures without mitochondria (Fig. 5 A) or with mitochondria (Fig. 5 B), in which Ca^{++} transport is abolished by the presence of inhibitors and uncouplers of respiration, produce identical absorbance decreases. This control confirms that in the above reported measurement of energy-linked Ca^{++} uptake, the amount of Ca^{++} bound to energy-independent sites is negligible, even at the lowest Ca^{++} concentrations. Furthermore Fig. 5, which shows that similar absorbance changes of murexide were obtained in the absence of succinate and phosphate, indicates that the presence of Mg^{++} and K^+ in the reaction mixtures prevents the formation of calcium phosphate or calcium succinate complexes in sizable amounts.

The initial velocity of Ca^{++} uptake by pigeon heart mitochondria is shown as a function of the added $[\text{Ca}^{++}]$ in Fig. 6. The titration curve is again sigmoidal in shape. In this case, the maximal velocity of Ca^{++} uptake occurred at about 300 μM Ca^{++} and the $[\text{Ca}^{++}]$ for half-maximal transport was about 65 μM . At any Ca^{++} concentration, the rate of Ca^{++} transport was diminished when pigeon heart mitochondria were isolated in the presence of the proteolytic enzyme Nagarse (41). This result confirms previous observations by Carafoli and Rossi (42), that Nagarse treatment interferes with the ability of heart mitochondria to transport Ca^{++} .

Fig. 7 shows the initial rates of energy-dependent Ca^{++} uptake by frog heart mitochondria as a function of the concentrations of added Ca^{++} . For

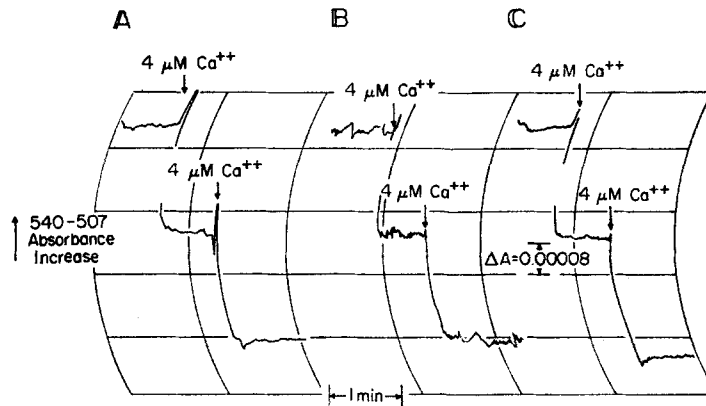


FIGURE 5. Absorbance changes of murexide upon addition of Ca^{++} to reaction mixtures with (Fig. 5 A) or without (Fig. 5 B and C) rat heart mitochondria. The reaction mixture contained 0.15 M sucrose, 0.075 M KCl, 5 mM MgCl_2 , and 55 μM murexide (Fig. 5 C). The mixtures were supplemented with 2 mM K_2HPO_4 and 1.7 mM Na succinate (Fig. 5 B), and with 2 mM K_2HPO_4 , 1.7 mM Na succinate, 5 μM FCCP, 10 μg antimycin A/ml, and 3.2 mg/ml of rat heart mitochondria in Fig. 5 A. Temperature was 24°C.

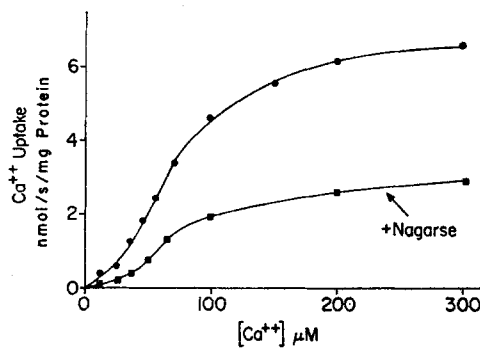


FIGURE 6

FIGURE 6. Initial velocity of Ca^{++} uptake by pigeon heart mitochondria at various Ca^{++} concentrations. Pigeon heart mitochondria were prepared either as described in the Methods or in the presence of Nagarse according to Chance and Hagihara (41). Ca^{++} uptake was measured as described in Fig. 3, except that in the reaction mixtures the concentration of mitochondria was 2.3 and 2.9 mg/ml for mitochondria prepared without or with Nagarse, respectively.

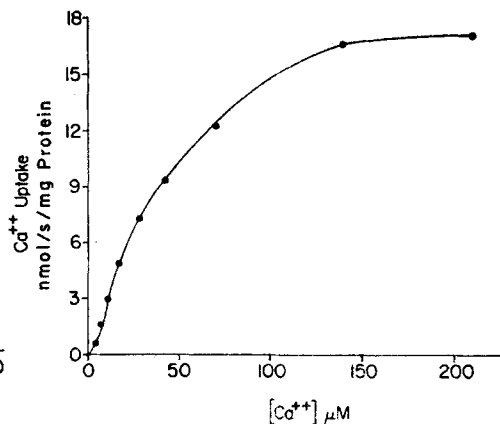


FIGURE 7

FIGURE 7. Titration of the initial rate of Ca^{++} uptake versus $[\text{Ca}^{++}]$ in frog heart mitochondria. Experimental conditions as in Fig. 3, except that the concentration of frog heart mitochondria was 3.1 mg/ml.

any given Ca^{++} concentration, the rate of Ca^{++} uptake was higher than with other heart mitochondria and the Ca^{++} concentration at which half-maximal Ca^{++} transport rate occurred was shifted toward lower Ca^{++} values. Similar findings were observed in all frog heart mitochondria preparations tested, and were independent of the size and the strain of frog from which the heart was taken. Although this difference could be in part attributed to sarcoplasmic reticulum contamination, this possibility is very unlikely because of the paucity of sarcoplasmic reticulum in heart, the way mitochondria were isolated, and the lack of effect of oxalate in enhancing Ca^{++} uptake.

Table II compares the values of energy-linked Ca^{++} uptake by mito-

TABLE II
ENERGY-LINKED Ca^{++} UPTAKE BY MITOCHONDRIA ISOLATED FROM DIFFERENT SOURCES

Source of mitochondria	Number of preparations tested	Initial velocities of Ca^{++} uptake		Ca^{++} concentration for half-maximal transport μM
		At 5 μM Ca^{++}	At 200 μM Ca^{++}	
		<i>nmol/s/mg</i>		
(a) Rat heart	11	0.04–0.09	8–14	85–105
(b) Guinea pig heart	3	0.03–0.07	7–12	80–95
(c) Squirrel heart	3	0.03–0.06	5–7	50–70
(d) Pigeon heart	4	0.06–0.13	6–9	60–85
(e) Pigeon heart (prepared with Nagarse)	3	0.01–0.06	2.5–3.5	65–75
(f) Frog heart	10	0.2–0.5	16–21	25–35

chondria isolated from hearts of various animal species. Initial velocities are reported at two Ca^{++} concentrations, one close to the V_{max} (200 μM) and one at 5 μM Ca^{++} . The latter concentration is the lowest measurable with accuracy by murexide methods and is slightly in excess of the reported concentration of Ca^{++} necessary for the saturation of cardiac myofibrils (1, 3, 6, 8). Although mitochondria from the heart of rat, guinea pig, squirrel, and pigeon all have comparable initial velocities of Ca^{++} uptake and apparent K_m for the Ca^{++} transport system, mitochondria from frog heart appear to have a more effective transport system for Ca^{++} .

DISCUSSION

Mitochondrial Ca^{++} Uptake and Heart Relaxation

The contraction-relaxation cycle of cardiac muscle, like that of skeletal muscle, is qualitatively controlled by the translocation of Ca^{++} among intracellular structures (1–7). It has been well established that the physiological relaxation of mammalian heart requires the removal of 50–100 nmol of Ca^{++} g/wt tissue from the troponin-binding sites in about 200 ms (1, 3, 5–7). Sarco-

plasmic reticulum, which is the source of Ca^{++} removal during the relaxation of skeletal muscle (1-5), is relatively scarce in heart (14, 15) and has a reduced ability to accumulate Ca^{++} in vivo (3, 5-7, 16). Therefore, the role of sarcoplasmic reticulum as the relaxing factor of the heart has been questioned by many investigators, and cardiac mitochondria have been suggested to regulate the contractile cycle of heart by taking up and releasing significant amounts of Ca^{++} during the cycle (9, 10, 12, 13).

Cardiac mitochondria have been shown to accumulate in vitro large amounts of Ca^{++} in an energy-linked process (9, 43, 44) and to lower the Ca^{++} concentration in the medium to about 10^{-7} M (45, 46), a concentration required for the relaxation of the myofibrils in vivo (3, 5, 6, 21). In addition, measurements of Ca^{++} distribution in subcellular fractions of the heart (10, 37) and the use of various inhibitors in perfused heart preparations (12, 13) have led to the conclusion that the energy-linked Ca^{++} movement of heart mitochondria plays a major role in the regulation of myocardial contractility. However, these and other studies have been regarded as too indirect and qualitative and have been criticized on the grounds of experimental approach and misinterpretation of the data obtained (3, 7, 18-20).

As indicated by Chance in early 1965 (43), the in vivo physiological role of mitochondria in regulating the beat-to-beat Ca^{++} cycle in heart must satisfy at least two requirements: thermodynamically mitochondrial Ca^{++} transport should be capable of accumulating high intraextramitochondrial Ca^{++} ratios, and kinetically Ca^{++} transport should operate with sufficient rapidity to participate in the Ca^{++} redistribution required by the contractile cycle. It is surprising that, in spite of its significance, the kinetic requirement for mitochondrial Ca^{++} uptake has not been verified by direct measurement. The affinity of mitochondrial Ca^{++} transport has been studied by measuring $^{45}\text{Ca}^{++}$ distribution after separation of mitochondria by centrifugation or filtration (9, 20, 43, 47), or by measuring events related to Ca^{++} uptake, such as changes in the oxygen consumption and shift in the redox state of either cytochrome *b* or flavoprotein (17, 43, 48). Alternatively, values of mitochondrial Ca^{++} apparent K_m have been estimated by equating the affinity of the Ca^{++} -transport system with that of the so called "high affinity Ca^{++} binding sites" in respiration inhibited mitochondria (49). These methods are rather indirect or obtained in ill-defined kinetic conditions and the value obtained for the apparent " K_m " of the mitochondrial Ca^{++} transport ranges from 0.1 to 50 μM Ca^{++} (1, 17, 43, 47-49). In the absence of an accurate value of the affinity of the Ca^{++} -transport system, the velocity of mitochondrial Ca^{++} uptake has often been calculated at saturating $[\text{Ca}^{++}]$, where the mitochondrial Ca^{++} uptake operates at relatively high rates. However, saturation of Ca^{++} transport in mitochondria occurs at a Ca^{++} concentration above 200 μM , which is presumably more than 100 times

greater than necessary for the contraction of the cardiac myofibrils (3–5, 6, 21, 56).

Initial Velocities of Mitochondrial Ca⁺⁺ Uptake

In this study, the direct measurement of the amount of Ca⁺⁺ taken up by isolated cardiac mitochondria during times comparable to that of cardiac relaxation was made using a stopped-flow technique and spectrophotometric detection of the change in absorbance by murexide (31). The properties of the metallochromic indicator, murexide, (high extinction coefficient [31], fast complexation rate with Ca⁺⁺ [50], extramitochondrial localization [30], lack of side effects on mitochondrial functions [30]), and the availability of sensitive spectrophotometric techniques (32) make possible fast detection of a 1–2 μM [Ca⁺⁺] transient in the presence of mitochondrial suspensions. Under our conditions, the amount of Ca⁺⁺ added to the reaction mixture is very close to the concentration of Ca⁺⁺ free in the medium. This condition was obtained (a) in the presence of Mg⁺⁺, which effectively competes with Ca⁺⁺ for the energy-independent Ca⁺⁺-binding sites in mitochondria (39, 40) and prevents the formation of calcium phosphate or calcium succinate in sizable amounts (*see* Fig. 5); and (b) by measuring the initial velocity of Ca⁺⁺ uptake after rapid Ca⁺⁺ mixing, in conditions where only a few percent of the Ca⁺⁺ added has been taken up by cardiac mitochondria. On the other hand, the number of sites for Ca⁺⁺ in the transport system is very small (49, 51) so that the amount of Ca⁺⁺ bound to these sites is negligible even at the lowest concentrations of Ca⁺⁺ added. Therefore, we assume that the reported system presents two major intrinsic advantages: it does not require the overcomplication of the EGTA-Ca⁺⁺ buffer to maintain constant the [Ca⁺⁺] in the medium, nor the arbitrary extrapolation of the data obtained in the initial rate values.

Our initial velocities of Ca⁺⁺ uptake are slightly higher than previous data obtained with ⁴⁵Ca⁺⁺ and separation of mitochondria through filtration and centrifugation (9, 11, 20, 43). This difference could be ascribed to the fact that we measured initial velocities and not the average of progressively decreasing velocities.

The Ca⁺⁺ concentration required for half-maximal activation of Ca⁺⁺ uptake was between 50 and 100 μM Ca⁺⁺. These values are one to two orders of magnitude higher than those recently reported by Carafoli and Azzi (17) and by Lehninger et al. (49) and closer to the value reported previously by Chance (43, 48). This discrepancy may be attributed to the way the measurements were made. Also in our system, the apparent K_m for Ca⁺⁺ was shifted toward lower [Ca⁺⁺] when, instead of the initial rates of Ca⁺⁺ uptake by murexide, either reduction of cytochrome *b* or the rate of oxygen consumption were measured. This brings into question the validity of measurements of

rates of oxygen uptake or redox state of cytochrome *b* as monitors of Ca^{++} transport and further studies may be necessary to clarify this point.

Cooperativity for Ca^{++} Transport and Physiological Significance

The sigmoidal plot of Ca^{++} uptake versus $[\text{Ca}^{++}]$ added, the linearity of the double-reciprocal plot when Ca^{++} uptake is plotted versus the square of $[\text{Ca}^{++}]$, and the Hill coefficient of 2 indicate that more than one molecule of Ca^{++} must be bound to the transport system to have Ca^{++} transported at significant rates. Cooperative interaction has been recently proposed for the binding of Na^+ and K^+ to the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ (52, 53), for the Ca^{++} uptake by sarcoplasmic reticulum (54, 55), for the ATP-dependent Ca^{++} uptake in rat liver mitochondria (47), and for the energy-dependent Ca^{++} and Mn^{++} uptake in liver mitochondria (36). We suggest that, as in liver mitochondria, the Ca^{++} -transport system located in the inner membrane of cardiac mitochondria has high efficiency only when two Ca^{++} are bound.

The high apparent K_m for Ca^{++} and the effect due to cooperativity make the Ca^{++} uptake very slow when the Ca^{++} concentration is below 10–15 μM . According to Weber and to Katz (1, 6), Ca^{++} begins to dissociate from the cardiac myofibrils at about 1 μM Ca^{++} and it is completely dissociated when the concentration of Ca^{++} is decreased to about 0.1 μM . Although the extrapolation of this value under in vivo conditions may present some uncertainty, this probably represents the range of values for cytosolic $[\text{Ca}^{++}]$ of cardiac cells, and evidence obtained with preparations of heart supports this figure (21, 56). There is no reason to assume that the cytosolic $[\text{Ca}^{++}]$ during contraction increases several times that required for the saturation of the myofibrils. Hence, the value of 5 μM , at which the velocity of Ca^{++} uptake by mitochondria was measured, should be considered in excess with respect to the cytosolic concentration of Ca^{++} in the heart. At 5 μM Ca^{++} , the uptake of Ca^{++} by rat heart mitochondria ranges from 0.05 to 0.13 nmol $\text{Ca}^{++}/\text{s}/\text{mg}$ mitochondrial protein at 25°C. On the basis of the reported temperature dependence of the Ca^{++} -transport system (43), the average duration of the heart relaxation period (6–8), and the amount of mitochondria present (cf. Table I) we calculate that only 0.9–1.3 nmol Ca^{++}/g wet cardiac tissue can be removed by mitochondria in vivo (38°C, 150 beats/min) during the relaxation time. This amount is two orders of magnitude less than the values of 50–160 nmol Ca^{++}/g wet tissue (8) which should be removed from the myofibrils during heart relaxation (1–3, 6–8). Similar estimates apply to guinea pig, squirrel, and pigeon hearts. The situation is slightly different in frog heart, where the mitochondrial Ca^{++} -transport system has a higher affinity for Ca^{++} and faster rates of Ca^{++} uptake. Due to these properties and the longer relaxation times of frog hearts (57), the mitochondria contained in 1 g wet frog heart should be able to take up to 6 nmol of Ca^{++} during the slower re-

laxation time. Therefore the amount of Ca⁺⁺ sequestered by frog heart mitochondria is still lower than the amount required for the relaxation of the myofibrils, but considerably higher than that sequestered by mitochondria in mammalian hearts. Evidence showing that frog heart is relatively deficient in sarcoplasmic reticulum (58, 59), and electrophysiological studies on frog hearts (57) have indicated that the overall cellular control of the beat-to-beat Ca⁺⁺ cycle in mammalian and in amphibian hearts may be considerably different (1, 6, 7). Whether in frog heart this control can be, in part or totally exercised by mitochondria, is debatable and further study is required.

In conclusion, if the extrapolation of *in vitro* data to *in vivo* conditions is permitted, and if the cytosolic Ca⁺⁺ concentration in heart is lower than 20 μ M, then the results indicate that the energy-linked Ca⁺⁺ uptake by mitochondria is grossly inadequate for the regulation of the beat-to-beat Ca⁺⁺ cycle of mammalian heart. Any further discussion of the overall physiological role of Ca⁺⁺ uptake by cardiac mitochondria remains speculative because of the lack of knowledge of the physiological significance of Ca⁺⁺ uptake by mitochondria in general. The reports showing that isolated mitochondria upon addition of both Ca⁺⁺ and ADP first transport Ca⁺⁺ and then phosphorylate ADP (43, 60), indicate that Ca⁺⁺ transport and phosphorylation are alternative processes in mitochondria. A cytosolic concentration of Ca⁺⁺ of 0.1–2 μ M and an allosteric interaction of Ca⁺⁺ in the transport system could accommodate both alternative functions of mitochondria within cardiac cells, ADP phosphorylation at physiological Ca⁺⁺ levels and Ca⁺⁺ transport when, in response to physiological or pathological events, the cytosolic concentration rises.

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