The Relationship between Free and Total Calcium Concentrations in the Matrix of Liver and Brain Mitochondria*

Susan Chalmers and David G. Nicholls‡

From the Buck Institute for Age Research, Novato, California 94945

Three sequential phases of mitochondrial calcium accumulation can be distinguished: matrix dehydrogenase regulation, buffering of extramitochondrial free calcium, and finally activation of the permeability transition. Relationships between these phases, free and total matrix calcium concentration, and phosphate concentration are investigated in rat liver and brain mitochondria. Slow, continuous calcium infusion is employed to avoid transient bioenergetic consequences of bolus additions. Liver and brain mitochondria undergo permeability transitions at precise matrix calcium loads that are independent of infusion rate. Cytochrome c release precedes the permeability transition. Cyclosporin A enhances the loading capacity in the presence or absence of acetocactate. A remarkably constant free matrix calcium concentration, in the range 1–5 μM as monitored by matrix-loaded fura2-FF, was observed when total matrix calcium was increased from 10 to at least 500 nmol of calcium/mg of protein. Increasing phosphate decreased both the free matrix calcium and the matrix calcium-loading capacity. Thus the permeability transition is not triggered by a critical matrix free calcium concentration. The rate of hydrogen peroxide detection by Amplex Red decreased during calcium infusion arguing against a role for oxidative stress in permeability pore activation in this model. A transition between a variable and buffered matrix free calcium concentration occurred at 10 nmol of total matrix calcium/mg protein. The solubility product of amorphous Ca₃(PO₄)₂ is consistent with the observed matrix free calcium concentration, and the matrix pH is proposed to play the major role in maintaining the low matrix free calcium concentration.

Isolated mitochondria from a variety of sources possess a large but finite capacity to accumulate and retain Ca²⁺ (for reviews see Refs. 1 and 2). When this limit is exceeded, mitochondria assemble a pore in their inner membrane that is non-selectively permeable to ions and solutes up to 1.4 kDa (for review see Ref. 3). It is unclear what exactly defines Ca²⁺, non-selectively permeable to ions and solutes up to 1.4 kDa (for review see Ref. 3). It is unclear what exactly defines Ca²⁺ non-selectivity. Ca²⁺ is a major intracellular ion with a multitude of metabolic functions. Its concentration in the cytosol is maintained at a relatively low level (100 nM) by an active transport systems across the plasma membrane and by the activity of the Na⁺/Ca²⁺ exchanger in the endoplasmic reticulum. In the cytosol, Ca²⁺ is sequestered in various organelles, such as the endoplasmic reticulum, the Golgi apparatus, and the lysosomes. In mitochondria, Ca²⁺ is accumulated during oxidative phosphorylation and is released during the permeability transition, leading to the induction of apoptosis. The permeability transition is a non-reversible change in mitochondrial membrane permeability that occurs at a critical matrix Ca²⁺ concentration and is characterized by the release of mitochondrial intermembrane space proteins, such as cytochrome c, and the depolarization of the mitochondrial inner membrane. The permeability transition is a key event in the regulation of mitochondrial function and survival.

The relationship between free and total matrix Ca²⁺ is crucial for the regulation of mitochondrial function. The permeability transition is a non-reversible change in mitochondrial membrane permeability that occurs at a critical matrix Ca²⁺ concentration and is characterized by the release of mitochondrial intermembrane space proteins, such as cytochrome c, and the depolarization of the mitochondrial inner membrane. The permeability transition is a key event in the regulation of mitochondrial function and survival. The activity of Ca²⁺ in the efflux pathways of both liver and brain mitochondria is inversely related to the free Pi concentration but independent of ΣCa²⁺/m, consistent with a matrix-free Ca²⁺, [Ca²⁺]m, that is governed by the solubility product of a calcium phosphate complex and is suboptimal for maximal activity of the efflux pathways of liver or brain mitochondria (4, 5). Third, mitochondria can maintain a remarkably constant setpoint when 2Ca²⁺/m is varied (6), indicating that the activity of the efflux pathway, and hence [Ca²⁺]m, is not varying. Fourth, mitochondria accumulating Ca²⁺ in the presence of excess phosphate extrude H⁺ per Ca²⁺ accumulated, consistent with the formation of Ca₃(PO₄)₂ in the matrix (7). Finally, Pi-depleted mitochondria in the presence of acetate as a permeant anion show an increasing efflux activity with increasing Ca²⁺ load and fail to maintain a setpoint that is independent of ΣCa²⁺/m (4).

All of these observations are consistent with a matrix Ca²⁺ phosphate complex that obeys mass-action relationships to maintain a low [Ca²⁺]m. However, any complex must be freely reversible, since the physiological complex is capable of dissociating into Ca²⁺ and P, immediately following mitochondrial depolarization, allowing the two ions to exit the mitochondrion on their individual carriers (5). Mitochondrial Ca²⁺ loading has almost invariably been investigated by the addition of one or more bolus additions of Ca²⁺, each of which induces a non-steady-state condition in which mitochondrial respiration, membrane potential and redox status change with time. Even multiple, relatively small, Ca²⁺ additions result in repetitive partial mitochondrial depolarizations and bioenergetic demands. In addition, it is highly likely that the calcium phosphate complex formed in response to a rapid increase in [Ca²⁺]m following a bolus addition may differ from that formed when Ca²⁺ is slowly accumulated, since many forms of calcium phosphate are thermodynamically unstable and spontaneously interconvert (8).

In this study we first investigate the capacity of liver and brain mitochondria to accumulate Ca²⁺ when the cation is slowly infused into the incubation, allowing the mitochondria to accumulate the cation continuously, with minimal bioenergetic demands. Second we monitor the matrix free Ca²⁺ concentrations during such loading in order to establish whether the supposed matrix buffering actually occurs, and finally we attempt to determine whether any relationship exists between matrix free Ca²⁺ and the initiation of the permeability transition.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 1-415-209-2095; Fax: 1-415-209-2232; E-mail: dnicolls@buckinstitute.org.

1 The abbreviations used are: ΣCa²⁺/m, total matrix Ca²⁺; [Ca²⁺]m, matrix free Ca²⁺ concentration; [Ca²⁺]e, extramitochondrial free Ca²⁺ concentration; Ksp, solubility product; CaG5N, Calcium Green 5N, TMRM, tetramethylrhodamine methyl ester; RLM, rat liver mitochondria; RBM, rat brain mitochondria; BSA, bovine serum albumin; Tes, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethylamino]ethanesulfonic acid.
Mitochondrial Free and Total Ca\(^{2+}\)

ANTIBODIES AND REAGENTS—Calcium Green 5N (CaG5N), fura-2-FF-AM, tetramethylrhodamine ester (TMRM), and Amplex Red were obtained from Molecular Probes (Eugene, OR). Anti-cytochrome c (7HB) was obtained from BD PharMingen (San Diego, CA). All other reagents were obtained from Sigma Chemical Co.

Preparation of Rat Liver Mitochondria (RLM)—Mitochondria were isolated from 6-week-old Wistar rats. The liver was homogenized in 125 mM sucrose, 125 mM mannitol, 5 mM Hepes, 1 mM EGTA, pH 7.2 and centrifuged at 1000 \(g\) for 10 min at 4 °C. The supernatant was then centrifuged for 10 min at 8000 \(g\) at 4 °C. The resulting pellet was resuspended and centrifuged for 10 min at 8500 \(g\) at 4 °C. The upper layer of this pellet was removed by gentle pipetting and the lower mitochondrial pellet resuspended in 0.5 ml of 250 mM sucrose, 16 mM BSA, 5 mM Hepes, pH 7.2.

Preparation of Rat Brain Mitochondria (RBM)—The cerebral cortices of two 6-week-old rats were rapidly removed into 20 ml of ice-cold isolation buffer (320 mM sucrose, 5 mM KCl, 1 mM EDTA, 10 mM NaHCO\(_3\), pH 7.4) and homogenized. The homogenate was centrifuged at 1000 \(g\) for 5 min at 4 °C. The supernatant was centrifuged at 8000 \(g\) for 10 min and the resulting pellet resuspended in 1 ml of isolation buffer. This was layered onto a discontinuous gradient consisting of 1 ml of 6% Ficoll, 0.5 ml of 250 mM sucrose, 16 mM KCl, 1 mM EDTA, 10 mM NaHCO\(_3\), pH 7.4, and 4.5 ml of 12% Ficoll (all prepared in isolation buffer) and centrifuged at 75,000 \(g\) for 30 min. The myelin, synaptosomal, and free mitochondrial fractions formed respectively above the 6% layer, as a doublet within the 9% layer and as a pellet. The pellet was resuspended in 250 mM sucrose, 16 mM bovine serum albumin, 10 mM Tes, pH 7.2 and centrifuged at 8000 \(g\) before being resuspended in the isolation buffer to 10–20 mg of protein/ml by the Bradford protein assay.

Mitochondrial Ca\(^{2+}\) Accumulation—0.1 mg of mitochondrial protein was suspended in 2 ml of incubation medium (100 mM NaCl, 25 mM Hepes, pH 7.2, 1 mM oligomycin, 2 mM sodium phosphate, and 0.5 mM ADP unless otherwise stated. A NaCl-based medium was used as this was shown in an earlier study (4) to give optimal respiratory control. Experiments with RLM utilized 2 mM succinate as substrate in the presence of 1 mM rotenone; those with RBM utilized 5 mM glutamate plus 5 mM malate or 5 mM pyruvate plus 5 mM malate. Experiments were performed at 37 °C in stirred cuvettes in a PerkinElmer LS-50B fluorimeter. CaCl\(_2\) additions were made either as bolus additions or as gradual infusions with a Braun Perfusor (FT Scientific Instruments, Glocester, UK) modified to take a Hamilton microsyringe (5).

Depletion of Endogenous Phosphate—Isolated mitochondria were depleted of endogenous phosphate by incubation with 1 mM glucose, 0.75 units/ml hexokinase, 1 mM MgCl\(_2\), 5 mM glutamate, 5 mM malate, and 0.5 mM ADP in incubation medium at 37 °C for 5 min in the presence of substrate as previously described (5).

Mitochondrial Free Ca\(^{2+}\)—RBM (0.1 mg of protein) were incubated in 25 \(\mu\)l of 250 mM sucrose, 10 mM Tes, 80 \(\mu\)M fura-2-FF AM, 16 mM BSA, pH 7.2 on ice for 30 min and then at room temperature for 30 min. Any contaminating synaptosomes were permeabilized by addition of 1 ml of 250 mM sucrose, 16 mM albumin, 5 mM Hepes, pH 7.2 containing 0.01% digitonin for the last 5 min of the incubation. The mitochondria were then centrifuged for 1 min at 10,000 \(g\), the pellet resuspended in 1 ml of 250 mM sucrose, 16 mM BSA, 5 mM Hepes, pH 7.2 and refuged. The pellet was finally resuspended in 20 \(\mu\)l of the same medium and used immediately.

Estimation of [Ca\(_{im}\)] in Equilibrium with Amorphous Tricalcium Phosphate as a Function of pH—The following dissociation constants were taken for phosphate, \(pK_1 = 2.13, pK_2 = 7.2, pK_3 = 12.39\) (10). A representative value for the \(K_\text{eq}\) for amorphous Ca3(PO4)2 was assumed, as published values vary from 10\(^{-26}\) to 10\(^{-33}\). This \(K_\text{eq}\) was assumed across the membrane via the phosphate carrier for an H\(_2\)PO\(_4^-\)/OH\(^-\) antipot (11). As the calculation was in -

**RESULTS**

Rat Liver Mitochondrial Ca\(^{2+}\)-loading Capacity during Steady Ca\(^{2+}\) Infusion—The capacity of RLM to accumulate Ca\(^{2+}\) in the presence of phosphate, and ADP is dependent upon the mode of addition (Fig. 1). A single bolus addition of 450 nmol of Ca\(^{2+}\)/mg initiates a permeability transition within 15 min, whereas 12 smaller additions of 50 nmol of Ca\(^{2+}\)/mg each (2.5 \(\mu\)mol each) or as a continuous infusion of 85 nmol/mg/min (42 \(\mu\)mol/min) (c).

**FIG. 1.** Effect of the mode of Ca\(^{2+}\) addition upon the capacity of liver mitochondria to accumulate Ca\(^{2+}\) before permeability transition initiation. 0.1 mg of RLM were suspended in 2 ml of incubation buffer (100 mM NaCl, 25 mM Hepes pH 7.2, 16 mM BSA, 2 mM NaPO\(_4\), 2 mM succinate, 0.2 mM ADP, 1 mM rotenone, 1 \(\mu\)g/ml oligomycin and 0.2 \(\mu\)g CaG5N). CaG5N was added as a single bolus addition of 450 nmol/mg (22.5 \(\mu\)mol) (a), as multiple additions of 50 nmol/mg each (2.5 \(\mu\)mol) (b), or as a continuous infusion of 85 nmol/mg/min (42 \(\mu\)mol/min) (c).
monitored in parallel with the Ca\(^{2+}\) fluorescence at 350/450 nm (c), swelling with light scattering at 625 nm (d), NAD(P) pool autofluorescence at 390/450 nm (c), or cytochrome c release by removing 100-μl samples (i–e) at the arrows (d), centrifuging to pellet the mitochondria and Western blotting the supernatants for cytochrome c. Note that the TMRM\(^{+}\) trace is inverted so that an upward deflection corresponds to an increase in membrane potential.

the cuvette. The onset of the permeability transition is signaled by the increase in [Ca\(^{2+}\)]

A would be predicted from the catastrophic consequences of removing the proton-impermeability of the inner membrane, permeability pore opening in individual mitochondria is associated with a virtually instantaneous collapse of membrane potential (12, 13). The resultant discharge of Ca\(^{2+}\) into the medium may trigger Ca\(^{2+}\) overload in adjacent mitochondria, leading to a chain reaction in which the entire mitochondrial population is seen to undergo the permeability transition in a short period of time. In order to minimize this effect in the present study, and also to ensure that anoxia did not occur during prolonged Ca\(^{2+}\) infusions, a very low mitochondrial protein concentration (0.05 mg/ml incubation) was used in most of these experiments.

Changes in mitochondrial membrane potential, NAD(P)H reduction, light scattering and cytochrome c retention were monitored in parallel with the Ca\(^{2+}\) infusion (Fig. 2). At the single mitochondrion level, the inner membrane permeabilization associated with the permeability transition results in an immediate collapse in membrane potential (13) and consequent loss of matrix Ca\(^{2+}\). A complicating factor therefore in conventional studies with mitochondrial populations is that this released Ca\(^{2+}\) can be taken up by adjacent mitochondria, either masking the initiation of the permeability transition, or triggering a chain reaction by inducing Ca\(^{2+}\) overload. Since the release of cytochrome c is an irreversible consequence of outer membrane rupture following the permeability transition, the experiment depicted in Fig. 2d, was performed to monitor the time course of release of the cytochrome during continuous infusion. Samples were taken from the cuvette during the Ca\(^{2+}\) infusion and rapidly centrifuged to pellet the mitochondria through silicone oil. Extensive cytochrome c release is first seen just before the massive release of matrix Ca\(^{2+}\) due to the permeability transition (sample iv). This suggests that a significant proportion of the mitochondria in the cuvette have undergone the transition by this stage. The modest increase in external free Ca\(^{2+}\) indicates that surrounding mitochondria accumulate Ca\(^{2+}\) from their depolarized neighbors, which would be predicted to facilitate in turn their depolarization and propagation of the transition throughout the incubation. A permeability transition-independent release of cytochrome c from rat brain mitochondria has been reported previously (14), although in that case it was induced by a single massive bolus addition of Ca\(^{2+}\) (3200 nmol/mg) and could therefore be due to transient osmotic swelling of the matrix prior to formation of the osmotically inactive calcium phosphate complex.

No change in the extent of TMRM\(^{+}\) quenching could be detected until shortly before the onset of the permeability transition (Fig. 2e). In contrast to Ca\(^{2+}\), whose distribution is the consequence of a dynamic balance between independent uptake and efflux pathways, TMRM\(^{+}\) responds in a Nernstian manner to the membrane potential. A permeability transition in a fraction of the mitochondria is therefore not masked by uptake of the membrane potential indicator by the residual mitochondria. For example, collapse in the membrane potential in 50% of the mitochondria would halve the aggregate matrix volume in which the TMRM\(^{+}\) is accumulated, releasing the probe and decreasing the quenching in the cuvette.

Light scattering increased steadily during the infusion (Fig. 2b). Since this parameter is a function of the difference in refractive index between the matrix and medium, it is likely that what is being observed is an increase in matrix refractive index due to the formation of a calcium phosphate complex rather than a physical contraction of the matrix (14).

NAD(P)H fluorescence also increases as Ca\(^{2+}\) is infused (Fig. 2c). As will be seen below (Fig. 8) [Ca\(^{2+}\)] \(_{im}\) is almost invariant over the range where the increased fluorescence signal is observed and so it is unlikely that the signal increases as a result of Ca\(^{2+}\)-dependent substrate dehydrogenase activation (15). Since fluorescence is sensitive to the environment, it is equally possible that the fluorescence change reflects a change in matrix environment rather than increased reduction.

By altering the speed of the infusion pump it is possible to determine whether the onset of the permeability transition is influenced by the rate or the extent of matrix Ca\(^{2+}\) loading. Varying the infusion rate from 42.5 to 170 nmol of Ca\(^{2+}\)/mg/min had no effect upon the capacity of the liver mitochondria to
accumulate Ca$^{2+}$ (Fig. 3a). In the presence of the permeability transition inhibitor cyclosporin A (Fig. 3b) the capacity increased almost 3-fold and again seemed independent of the rate of infusion, although a slight decrease in capacity was observed for the slowest infusion.

Oxidation of RLM matrix NADH by acetoacetate in the absence of exogenous adenine nucleotides facilitates the permeability transition in response to bolus additions of Ca$^{2+}$ (16). It has recently been reported (17) that acetoacetate activates a low conductance form of the permeability transition pore in RLM, that is sensitive to cyclosporin A and manifests itself as an increase in the rate of Ca$^{2+}$ efflux observed following ruthenium red addition, but without a detectable loss of membrane potential. Under the present conditions, NADH oxidation induced by acetoacetate does not increase the steady-state rate of Ca$^{2+}$ efflux from the mitochondria, since that would be reflected in an increased steady-state [Ca$^{2+}$]$_m$, and none can be detected (Fig. 4), but instead the capacity of the mitochondria to accumulate Ca$^{2+}$ is considerably decreased. Importantly this effect of acetoacetate is still observed in the presence of 1 mM cyclosporin and ADP (Fig. 4).

**Fig. 4.** Effect of acetoacetate upon mitochondrial Ca$^{2+}$ accumulation capacity. Rat liver mitochondria were suspended and infused with Ca$^{2+}$ as in Fig. 1c with the inclusion of 1 mM CsA where shown. 2 mM lithium acetate was added 2 min after the start of Ca$^{2+}$ infusion (arrow) where shown (AA).

Relationship between Free and Total Matrix Ca$^{2+}$ Concentrations during Ca$^{2+}$ Infusion—Preincubation of isolated mitochondria with fluorescent Ca$^{2+}$ indicators can allow sufficient loading to occur to monitor matrix free Ca$^{2+}$ concentrations (15, 18–24). In our hands it was difficult to achieve a reliable extent of loading with RLM and so subsequent experiments were performed with RBM.

The low affinity fura analog fura2-FF ($K_d$ = 5.5 µM) was found to load into the mitochondrial matrix as the acetyloxymethyl ester and to be hydrolyzed sufficiently to enable changes in [Ca$^{2+}$]$_m$ to be followed. Since mitochondrial autofluorescence, due primarily to NAD(P)H at the wavelengths employed, changes at the onset of the permeability transition (Fig. 2) in most experiments this was corrected for by parallel determinations in the absence of loaded dye.

The hypothesis on which these studies were based was that changes in [Ca$^{2+}$]$_m$ in the presence of excess phosphate would be largely buffered by the formation of calcium phosphate complexes. In order initially to assess the ability of the loaded fura2-FF to detect changes in [Ca$^{2+}$]$_m$ therefore, mitochondria were extensively depleted of endogenous Pi by preincubation with ADP, glucose and hexokinase, as previously described (5), and acetate was added as permissive anion to prevent build up of a pH gradient. Since calcium acetate is highly soluble it would be predicted that [Ca$^{2+}$]$_m$ would rise with Ca$^{2+}$ load. Fig. 5 shows the increase in [Ca$^{2+}$]$_m$ of RLM loaded with fura2-FF following four bolus additions of 10 µM Ca$^{2+}$ to the cuvette. It is notable that the matrix free Ca$^{2+}$ rose stepwise with each addition. In contrast, [Ca$^{2+}$]$_m$, determined in parallel with external CaG5N, partially recovered after each bolus addition as Ca$^{2+}$ was accumulated into the matrix. Subsequent addition of 1 µM FCCP to depolarize the mitochondria resulted in an extrusion of Ca$^{2+}$ from the matrix to the suspension medium, a decrease in [Ca$^{2+}$]$_m$ and increase in [Ca$^{2+}$]$_e$, corresponding to Ca$^{2+}$ efflux via the unipporter.

There is compelling evidence that the independent Ca$^{2+}$ efflux pathway in heart mitochondria (15, 22) is not saturated by [Ca$^{2+}$]$_m$ under conditions of limited Ca$^{2+}$ loading when Ca$^{2+}$-dependent changes in dehydrogenase activation can be observed. Thus the steady-state [Ca$^{2+}$]$_m$ determined both from matrix-loaded fura2 and indirectly by the activation state of
matrix dehydrogenases increases with \([Ca^{2+}]_m\), (15) while under extensive loading conditions the activity of the efflux pathways is invariant with matrix \(Ca^{2+}\) load but varies inversely with the matrix free phosphate (5).

Fig. 6 shows the transition between these two phases of matrix \(Ca^{2+}\) loading. When isolated mitochondria are suspended in incubation media similar to that used here, there is sufficient contaminating \(Ca^{2+}\) in the medium and residual \(Ca^{2+}\) in the matrix such that the initial loading condition when \([Ca^{2+}]_m\) varies with \(Ca^{2+}\) load is not seen. On addition of 10–30 \(\mu M\) EGTA, a sharp decline in the steady-state \([Ca^{2+}]_m\) was observed as \(\Sigma Ca_m\) decreased from 10 to 3 nmol/mg (Fig. 6), consistent with a decrease in free matrix \(Ca^{2+}\) (5). In contrast, additions of \(Ca^{2+}\) caused little increase in steady-state \([Ca^{2+}]_m\), indicating that the setpoint had been attained.

Since inefficiency of the \([Ca^{2+}]_m\) buffering in the presence of acetate contrasts so much with that seen in the presence of excess \(P_i\) (Fig. 1), the inference is that the latter allows \([Ca^{2+}]_m\) to be essentially independent of \(\Sigma Ca_m\) over the range where a setpoint is observed, in the present preparation from 20 nmol/mg (Fig. 6) to 500 nmol/mg \(\Sigma Ca_m\) (Fig. 1). Studies to investigate this were performed on RBM. Since RBM have been measured fluorimetrically. The \([Ca^{2+}]_m\) was steadily infused at a rate of 85 nmol/min/mg, while \([Ca^{2+}]_m\) was monitored with CaGreen-5N and \([Ca^{2+}]_m\) by fura-2-FF.

Three phases of \([Ca^{2+}]_m\) maintenance can be distinguished in the presence of 5 mM \(P_i\). No increase in \([Ca^{2+}]_m\) was detected during the presence of the first 120 nmol/mg of \(Ca^{2+}\). For the next 360 nmol/mg \(Ca^{2+}\) infusion a slow increase in \([Ca^{2+}]_m\) can be observed, while above a \(2Ca^{2+}_m\) of 480 nmol/mg \([Ca^{2+}]_m\) starts to increase at a rate comparable to that seen initially in \(P_i\)-depleted mitochondria (not shown), where \([Ca^{2+}]_m\) increases immediately and uniformly.

The discontinuity in the \([Ca^{2+}]_m\) trace after infusion of 480 nmol/mg corresponds to the point at which \([Ca^{2+}]_m\) monitored by CaGreen5N starts to increase (Fig. 8b), and this may represent the initiation of the permeability transition, which would allow matrix fura2-FF to be released and equilibrate with \([Ca^{2+}]_m\). The relationship between \([Ca^{2+}]_m\) and \(\Sigma Ca^{2+}_m\) is shown in more detail in Fig. 8c. Based on a matrix volume of 1 \(\mu l/mg\) the ratio of bound/free \(Ca^{2+}\) in the matrix can reach 150,000 in \(Ca^{2+}\)-loaded rat brain mitochondria.

It is frequently assumed that the permeability transition is triggered by an increase in matrix free \(Ca^{2+}\), however increasing external phosphate decreases the capacity of mitochondria to accumulate \(Ca^{2+}\) before the permeability transition is activated (25). If the assumption is correct that \([Ca^{2+}]_m\) is defined by the solubility product of a calcium phosphate complex, then \([Ca^{2+}]_m\) would be buffered at lower values by the increased matrix \(P_i\). This is confirmed in Fig. 9. Increasing external \(P_i\) from 2 to 5 mM decreases the capacity of brain mitochondria to buffer \([Ca^{2+}]_m\) by almost 50%; however, the \([Ca^{2+}]_m\) at which the permeability transition was activated in the presence of 5 mM \(P_i\) was substantially lower than that in the presence of 2 mM \(P_i\). It can be concluded that the permeability transition is not triggered by an increased \([Ca^{2+}]_m\). Consistent with this, cyclosporin A has no effect on the relationship between \([Ca^{2+}]_m\) and \(\Sigma Ca^{2+}_m\) during \(Ca^{2+}\) infusion (Fig. 10). Cyclosporin A more than doubled the capacity of the mitochondria to accumulate and retain \(Ca^{2+}\) without affecting the relationship between \(\Sigma Ca^{2+}_m\) and \([Ca^{2+}]_m\) until the permeability transition is activated.

The release of hydrogen peroxide from brain mitochondria oxidizing NAD-linked substrates is membrane potential-dependent, and bolus additions of \(Ca^{2+}\) decrease \(H_2O_2\) release monitored by Amplex Red to an extent that can be correlated with a decrease in membrane potential (26). Since slow \(Ca^{2+}\)
infusion occurs independently of any significant depolarization (Fig. 2), H2O2-dependent Amplex Red oxidation in the presence of cyclosporin A was monitored during infusion of Ca2+/H11001 (Fig. 11). Compared with a parallel blank infusion there was a slow decline in the rate. It is thus not evident that the permeability transition can be ascribed to a Ca2+-dependent oxidative stress.

DISCUSSION

Matrix Free Ca2+/H11001—A series of studies performed 10–15 years ago established that free Ca2+/H11001 concentrations within the matrices of isolated liver and heart mitochondria varied over a range from 0.1 to 2 μM under conditions of limited matrix Ca2+/H11001 loading, i.e. <10 nmol/min/mg of protein (15, 18–23, 27). Three approaches were taken, a null-point technique in which extramitochondrial free Ca2+/H11001 was adjusted until the addition of the Ca2+/H11001 ionophore A23187 caused no net flux of Ca 2+/H11001 (18, 28), determination from the activation state of Ca2+/H11001-dependent matrix dehydrogenases (15, 23) and loading or entrapment of optical Ca2+/H11001 indicators (15, 19, 20, 23, 27). Under these conditions matrix free Ca2+/H11001 varied with external free Ca2+/H11001 in a way consistent with physiological control of matrix dehydrogenase activities (for review see Ref. 29).

In recent years there have been numerous studies with both intact and digitonin-permeabilized cells that have been loaded with the cationic Ca2+/H11001 indicator Rhod-2. Under appropriate conditions the positive charge localizes the ester to the mitochondrial matrix where it is hydrolyzed to generate Rhod-2, a Ca2+/H11001 indicator with a Kd of 0.57 μM. The ability of this high affinity probe to detect changes in [Ca 2+/H11001]m within intact cells indicates that this parameter can vary in situ over the range where Rhod-2 is responsive, say 0.06–6 μM. Substantially higher estimates of transient [Ca2+]m elevations have been
Mitochondrial Free and Total Ca\(^{2+}\)

Matrix Total Ca\(^{2+}\)—A limitation with the above studies is that total Ca\(^{2+}\) is generally not determined in parallel, and so it is not possible to draw conclusions about the chelation of matrix Ca\(^{2+}\) or how this changes with matrix load. In 1978 the first studies were performed in which steady-state extramitochondrial free Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)\(_{ext}\)]) were monitored during extensive matrix Ca\(^{2+}\) loading (4, 5, 31). In the presence of excess P\(_i\) and physiological concentrations of adenine nucleotides, liver (6), and brain (4) mitochondria were able to restore a [Ca\(^{2+}\)\(_{int}\)] that was independent of matrix Ca\(^{2+}\) load from 10 to almost 1000 nmol of Ca\(^{2+}\)/mg.

Analysis of the individual kinetics of the Ca\(^{2+}\) uniporter and efflux pathways indicated that this setpoint was the consequence of a kinetic balance between the activities of a uniporter highly dependent upon [Ca\(^{2+}\)\(_{int}\)], and an efflux pathway activity apparently totally insensitive to changes in total matrix Ca\(^{2+}\) over this range (5). This latter in turn could be due either to saturation of the efflux pathway by [Ca\(^{2+}\)\(_{int}\)] or to a [Ca\(^{2+}\)\(_{int}\)] that was essentially independent of matrix Ca\(^{2+}\) load over this range. Two experiments supported the latter alternative and indicated that complexation of Ca\(^{2+}\) with matrix P\(_i\) helped to maintain a constant [Ca\(^{2+}\)\(_{int}\)]. First the activity of the RLM efflux pathway was inversely related to the P\(_i\) concentration (5) and second when acetate replaced P\(_i\) as permeant anion the Ca\(^{2+}\) efflux activity, and hence the setpoint increased continuously with matrix load (5).

While such studies were initially criticized as being non-physiological, the advent of digital Ca\(^{2+}\) imaging demonstrated that cytoplasmic free Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{cyt}\)]) in excitable cells undergoes large excursions far exceeding the setpoint values obtained with isolated mitochondria (33), and it is now established that in situ mitochondria can accumulate large amounts of Ca\(^{2+}\) when the local [Ca\(^{2+}\)\(_{cyt}\)] is above 0.5 \(\mu\)M and release Ca\(^{2+}\) to the cytoplasm when [Ca\(^{2+}\)\(_{cyt}\)] is restored to basal values (e.g., Ref. 34). It is however apparent that monitoring [Ca\(^{2+}\)\(_{int}\)] of in situ mitochondria provides little information on the extent of total Ca\(^{2+}\) accumulation within the matrix. Thus in repetitively stimulated lizard motor nerve terminals the sustained clearane of cytoplasmic Ca\(^{2+}\) by mitochondria fails to increase [Ca\(^{2+}\)\(_{int}\)] above 1 \(\mu\)M regardless of stimulation frequency or the amount of accumulated Ca\(^{2+}\) (35). The present study shows (Fig. 8) that [Ca\(^{2+}\)\(_{int}\)] of isolated rat brain mitochondria changes by less than 20\% when total matrix Ca\(^{2+}\) is increased from about 10 to 400 nmol/mg.

These two aspects of mitochondrial Ca\(^{2+}\) accumulation are not mutually incompatible, and as shown in Fig. 6, there is a smooth transition from the region where [Ca\(^{2+}\)\(_{int}\)] varies with Ca\(^{2+}\) load to the invariant region associated with a constant setpoint and buffered [Ca\(^{2+}\)\(_{int}\)].

The Nature of the Matrix Calcium Phosphate Complex—Although much evidence points to the presence of a calcium phosphate complex within the matrix of loaded mitochondria, its nature remains obscure, due largely to the considerable complexity and variability of biologically relevant calcium phosphate forms (for review see Ref. 10). Additionally, investigations are hindered by the ability of the presumed matrix complex instantly to dissociate into Ca\(^{2+}\) and P\(_i\) when the Ca\(^{2+}\)-loaded mitochondria are depolarized, so that Ca\(^{2+}\) exits via the reversal of the uniporter, and P\(_i\) exits via the P\(_i\) transporter (5).

The formation of a calcium phosphate complex within the matrix that is in equilibrium with 5 mm free phosphate but with only 2 \(\mu\)M [Ca\(^{2+}\)\(_{int}\)] (Fig. 8) has to be reconciled with the ability to prepare physiological extracellular buffers containing

---

**Fig. 10. Effect of CsA upon [Ca\(^{2+}\)\(_{int}\)]** Fura2-FF-loaded RBM were suspended as in Fig. 1 with the addition of 1 \(\mu\)M Amplex Red plus 0.75 units/ml horseradish peroxidase. 85 nmol/mg Ca\(^{2+}\) was infused as shown and [Ca\(^{2+}\)\(_{int}\)] and [Ca\(^{2+}\)\(_{ext}\)] monitored simultaneously.

**Fig. 11. Effect of gradual Ca\(^{2+}\) accumulation on the rate of H\(_2\)O\(_2\) production.** Rat liver mitochondria were suspended as in Fig. 1 with the addition of 1 \(\mu\)M Amplex Red plus 0.75 units/ml horseradish peroxidase. 85 nmol/mg Ca\(^{2+}\) (+Ca\(^{2+}\)) or an equal volume of H\(_2\)O (no Ca\(^{2+}\)) was then infused and H\(_2\)O\(_2\) monitored as an increase in 563/587 nm fluorescence — lower traces. Each line is an average of three experiments. An additional infusion was carried out with 0.2 \(\mu\)M CaGSK in place of Amplex Red plus horseradish peroxidase to indicate the point of PT — upper trace, offset upwards by 10 units for clarity.

---

reported using matrix-targeted aequorins (30), although the in vitro calibration technique employed has recently been criticized (31), since with an in situ calibration, values close to those obtained with Rhod-2 have been obtained (31). However, since aequorin is consumed by Ca\(^{2+}\), it is not suitable for the measurement of long term steady-state concentrations as studied here.
Mitochondrial Free and Total Ca$^{2+}$

**Fig. 12. Possible stoichiometries of Ca-P complex formation in the mitochondrial matrix.** Ion transport stoichiometries are shown for respiration-linked mitochondrial Ca$^{2+}$ accumulation in the presence of excess phosphate. The external pH is assumed to be 7.2 such that there are equal concentrations of H$_3$PO$_4$ and HPO$_4^{2-}$. The phosphate carrier is assumed to function as a H$_3$PO$_4$ uniporter, this is equivalent to a H$_2$PO$_4^-$/OH⁻ antiport. a, amorphous tricalcium phosphate, Ca$_3$(PO$_4$)$_2$, forms in the matrix, a H$^+$/Ca$^{2+}$ ratio of 1 is seen in the external medium. b, CaHPO$_4$ formation gives a H$^+$/Ca$^{2+}$ ratio of 0.5. c, CaHPO$_4$$_2$ formation gives a H$^+$/Ca$^{2+}$ ratio of 1. d, hydroxyapatite formation would be associated with a H$^+$/Ca$^{2+}$ ratio of 1.1. e, matrix free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_m$) in equilibrium with either amorphous tricalcium phosphate (TCA, $K_{sp} = 3 \times 10^{-59}$) or hydroxyapatite (HAP, $K_{sp}$ = $1 \times 10^{-59}$) for two concentrations of total external phosphate (2 and 5 mM) and as a function of matrix pH. The three dissociation constants for phosphate were taken as: $p$$K_1$ = 2.13, $p$$K_2$ = 7.2, $p$$K_3$ = 12.39 (10). Note that the gradient of the PO$_4^{3-}$ anion varies as the third power of the pH gradient across the inner membrane, thus matrix acidification from pH 8.0 to pH 7.0 would decrease the matrix PO$_4^{3-}$ concentration by a factor of 1000. At a constant solubility product for Ca$_3$(PO$_4$)$_2$, this would raise the [Ca$^{2+}$]$_m$ in equilibrium with the complex by 100-fold. In the presence of 5 mM total P, an indicated [Ca$^{2+}$]$_m$ of 2.1 $\mu$M would correspond to a matrix pH close to 7.7, i.e. 0.5 pH units alkaline with respect to the medium.

Similar phosphate concentrations but one-thousand times higher free Ca$^{2+}$ concentrations. Similarly, external Ca$^{2+}$ electrodes or low affinity extramitochondrial Ca$^{2+}$ indicators show that Ca$^{2+}$ released from Ca$^{2+}$-loaded mitochondria into P$_r$-containing media remains in solution even though the [Ca$^{2+}$] is much higher than the [Ca$^{2+}$]$_m$ indicated by the matrix-loaded fura2-FF (Figs. 9 and 10). The contrast between the ability of polarized mitochondria to retain enormous amounts of Ca$^{2+}$ within their matrices and the rapid and almost complete release of the ion when the mitochondria are depolarized is additionally puzzling since the concentration gradients of free Ca$^{2+}$ and P$_r$ across the polarized membrane appear to be so small (Fig. 8). [Ca$^{2+}$]$_m$, being limited by the solubility product of the complex and [P$_r$]$_m$ being defined from the external P$_r$ and the transmembrane pH gradient.

Hydroxyapatite can be detected in fixed and desiccated samples, but it is generally accepted that this is an artifact (36). In an artificial cytoplasm in the presence of ATP, amorphous Ca$_3$(PO$_4$)$_2$ is initially formed when millimolar Ca$^{2+}$ is titrated, particularly at pH 7.5–8.0 (corresponding to the matrix pH) and is stable for prolonged periods (8). At external pH 7.0, the mitochondrial uptake of one Ca$^{2+}$ in the presence of excess P$_r$ is accompanied by the net extrusion of one proton (7), consistent with the rapid formation of Ca$_3$(PO$_4$)$_2$ (Fig. 12).

Reported values for the ion activity product (solubility product) of amorphous Ca$_3$(PO$_4$)$_2$ differ widely in the literature, from $2 \times 10^{-33}$ to $1.6 \times 10^{-25}$. It is not easy to find the original references in which these constants were determined, and in any case the mitochondrial matrix is perhaps as far from an ideal solution as it is possible to imagine, but nevertheless the solubility properties of this calcium salt provides a way to understand a number of apparently conflicting aspects of calcium storage in the mitochondrial matrix. Thus it is not immediately apparent how micromolar free Ca$^{2+}$ concentrations could form such a complex with P$_r$ within the mitochondrial matrix, while much higher free Ca$^{2+}$ concentrations can coexist with millimolar P$_r$ in physiological incubation media without precipitation. Secondly, if the gradient of free Ca$^{2+}$ concentration across the inner membrane is really so small (1–4-fold) as is indicated by this and other studies, why is a high membrane potential required to retain Ca$^{2+}$ within the matrix and why does the addition of protonophore lead to such a massive and rapid efflux of Ca$^{2+}$ and P$_r$? The answer may lie in the high pH dependence of the Ca$^{2+}$ concentration that is in equilibrium with amorphous Ca$_3$(PO$_4$)$_2$ (Fig. 12). The highly active phosphate carrier equilibrates the transported species H$_2$PO$_4^-$ with OH$^-$ (11) and thus accumulates the monoaion as a function of ΔpH. The further dissociations of H$_2$PO$_4^-$ to HPO$_4^{2-}$, and of HPO$_4^{2-}$ to PO$_4^{3-}$, the species that complexes with Ca$^{2+}$ to form Ca$_3$(PO$_4$)$_2$, are each dependent on pH$_m$, with the result that the concentration of the PO$_4^{3-}$ species increases as the third power of pH$_m$. Maintenance of the solubility product for Ca$_3$(PO$_4$)$_2$ thus means that the concentration of free matrix Ca$^{2+}$ in equilibrium with the complex decreases as the second power of the matrix pH and as the two-thirds power of the total external phosphate concentration (Fig. 12). The collapse or even tempo-
rinary reversal of $\Delta pH$ following addition of a protonophore would thus be predicted to facilitate dissociation of the complex, liberating free $\text{Ca}^{2+}$ and facilitating its rapid efflux via reversal of the uniporter in response to the concomitant collapse of $\Delta \psi_m$.

The Relationship between Bolus $\text{Ca}^{2+}$ Additions and Continuous Infusion—This is the second study in which we have utilized slow, continuous $\text{Ca}^{2+}$ infusion in order to investigate mitochondrial $\text{Ca}^{2+}$ transport. In the first (5) we determined the kinetics of the rat liver mitochondrial $\text{Ca}^{2+}$ uniporter by infusing the cation at different rates and determining the value at which $[\text{Ca}^{2+}]_i$ stabilized, i.e., when the uniporter activity exactly balanced that of the infusion rate plus the activity of the efflux pathway. Uniporter activity was found to increase as an exponential function of $[\text{Ca}^{2+}]_i$ until respiration became rate-limiting (5).

It is extraordinarily complex to analyze mitochondrial $\text{Ca}^{2+}$ transport and sequestration in response to a bolus addition of $\text{Ca}^{2+}$—loading capacity of brain mitochondria. Two scenarios may be envisaged, one in which $\text{Ca}^{2+}$ entry and is initiated by the enhanced transmembrane pH gradient.

Relevance to in Vivo Mitochondrial $\text{Ca}^{2+}$ Transport—It is becoming increasingly apparent that brain mitochondria in situ can accumulate $\text{Ca}^{2+}$ under a variety of physiological and pathological conditions such as epilepsy (40), ischemia (41, 42), and concussive brain injury (43). At the same time there is active debate as to whether the permeability transition participates in neuronal ischemic death and whether permeability transition inhibitors are neuroprotective (9, 32, 37, 44–46). It is clearly important to establish the factors that define the $\text{Ca}^{2+}$-loading capacity of brain mitochondria. Two scenarios may be envisaged, one in which in situ brain mitochondria load $\text{Ca}^{2+}$ rapidly in response to a sudden dramatic increase in free cytoplasmic $\text{Ca}^{2+}$, for example due to repetitive firing of voltage-activated $\text{Ca}^{2+}$ channels, and one in which they slowly accumulate the cation in response to a slow uncompensated inward leak of the cation across the plasma membrane. The present study may be of relevance to the latter. It is apparent that phosphate plays a major role in defining the capacity of the mitochondria; it would of importance to determine whether cytoplasmic phosphate is in excess or is limiting under the above conditions of mitochondrial $\text{Ca}^{2+}$ loading.

REFERENCES

1. Nicholls, D. G., and Åkerman, K. E. O. (1982) Biochim. Biophys. Acta 683, 57–88.
2. Gunter, T. E., Gunter, K. K., Sheu, S.-S., and Gavin, C. E. (1994) Am. J. Physiol. Cell Physiol. 267, C313–C339.
3. Bernardi, P. (1999) Physiol. Rev. 79, 1127–1155.
4. Nicholls, D. G., and Scott, I. D. (1980) Biochem. J. 186, 833–839.
5. Zoccarato, F., and Nicholls, D. G. (1982) Eur. J. Biochem. 127, 333–338.
6. Nicholls, D. G. (1978) Biochem. J. 176, 463–474.
7. Lehninger, A. L., Carafoli, E., and Rossi, C. S. (1967) Adv. Enzymol. Relat. Areas Mol. Biol. 29, 259–320.
8. Wiederrecht, R. E., Rice, G. S., Wallace, J. E., Weaver, R. L., LeGeros, R. Z., and Eanes, E. D. (1985) Calcif. Tissue Int. 37, 401–410.
9. Friberg, H., Ferrand-Drake, M., Bengtsson, F., Halseström, A. P., and Wieloch, T. (1998) J. Neurosci. 18, 5151–5159.
10. Larsen, M. J. (1986) Arch. Oral Biol. 31, 757–761.
11. Kramer, R. (1988) Exp Physiol 83, 259–265.
12. Huser, J., Rechmann, C. E., and Blatter, L. A. (1998) Biophys. J. 74, 2129–2137.
13. Huser, J., and Blatter, L. A. (1999) Biochem. J. 343, 311–317.
14. Andreyev, A., Fahy, E., and Fiskum, G. (1998) FEBS Lett. 439, 373–376.
15. Moreno, S., and Hansford, R. G. (1986) Biochem. J. 256, 403–412.
16. Nicholls, D. G., and Brand, M. D. (1980) Biochem. J. 186, 113–118.
17. Zago, E. B., Castilho, R. F., and Vercesi, A. E. (2000) FEBS Lett. 478, 29–33.
18. Hansford, R. G., and Castro, P. (1982) J. Bioenerg. Biomembr. 14, 361–376.
19. Davis, M. H., Altschuld, R. A., Jung, D. W., and Brierley, G. P. (1987) Biochim. Biophys. Res. Commun. 149, 40–45.
20. Łukacisz, G. L., and Kapus, A. (1987) Biochem. J. 246, 609–613.
21. Gunter, T. E., Restrepo, D., and Gunter, K. K. (1998) Am. J. Physiol. 250, C304–C310.
22. Łukacisz, G. L., Kapus, A., and Fonyo, A. (1988) FEBS Lett. 229, 219–223.
23. McCormack, J. G., Browne, H. M., and Dawes, N. J. (1989) Biochim. Biophys. Acta 974, 420–427.
24. Allen, S. P., Stone, D., and McCormack, J. G. (1992) J. Mol. Cell Cardiol. 24, 765–773.
25. Zotti, M., and Szabo, I. (1995) Biochim. Biophys. Acta 1241, 139–176.
26. Starovk, A. A., Polster, B. M., and Fiskum, G. (2002) J. Neurochem. 83, 220–228.
27. Al-N, and Crompton, M. (1986) Biochem. J. 239, 31–40.
28. Coll, K. E., Joseph, S. K., Corkey, B. E., and Williamson, J. R. (1982) J. Biol. Chem. 257, 6129–6134.
29. Zaidan, E., and Sims, N. R. (1994) Brain Res. 639, 216–219.
30. Bizzari, R., Punton, P., Brion, M., Chessa, A., Filippini, L., and Pozzan, T. (1999) Cell Calc. 26, 193–199.
31. Frier, J. G., Maechler, P., Wollheim, C. B., and Spat, A. (2002) Cell Calc. 31, 279–294.
32. Frier, H., Conner, C., Halseström, A. P., and Wieloch, T. (1999) J. Neurochem. 72, 2488–2497.
33. Connors, J. A. (1980) Proc. Natl. Acad. Sci. U. S. A. 83, 6179–6183.
34. Wang, G. J., and Thayer, S. A. (1996) J. Neurophysiol. 76, 1611–1621.
35. David, G. (1999) J. Neurosci. 19, 7495–7506.
36. Larre, J. P., Thoren, A., and Jansen, S. J. S. (1985) Calcif. Tissue Int. 37, 169–180.
37. Nakatsuka, H., Ohta, S., Tanaka, J., Toku, K., Kumon, Y., Maeda, N., Sakamura, G., and Saka, S. (1999) Brain Res. 849, 216–219.
38. Andreyev, A., and Fiskum, G. (1999) Cell Death Differ. 6, 825–832.
39. Bernardi, P. (1996) Biochim. Biophys. Acta Bio-Energetics 1275, 5–9.
40. Griffiths, T., Evans, M. C., and Meldrum, B. S. (1982) Neurosci. Lett. 30, 329–334.
41. Erecinska, M., and Silver, I. A. (1992) Can. J. Physiol. Pharmacol. 70, suppl.)
S190–S193.
42. Zaidan, E., and Sims, N. R. (1994) J. Neurochem. 63, 1812–1819.
43. Frier, H., Conner, C., Halsestrom, A. P., and Wieloch, T. (1999) J. Cereb. Blood Flow Metab. 19, 736–741.
44. Folbergrová, J., Li, P. A., Uchino, H., Smith, M. L., and Siesjö, B. K. (1997) Exp. Brain Res. 114, 44–50.
45. Li, P. A., Uchino, H., Elmer, E., and Siesjö, B. K. (1997) Brain Res. 753, 133–140.
The Relationship between Free and Total Calcium Concentrations in the Matrix of Liver and Brain Mitochondria
Susan Chalmers and David G. Nicholls

J. Biol. Chem. 2003, 278:19062-19070.
doi: 10.1074/jbc.M212661200 originally published online March 26, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212661200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 43 references, 8 of which can be accessed free at http://www.jbc.org/content/278/21/19062.full.html#ref-list-1