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Transport of Calcium by Mitochondria

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The identification of intramitochondrial free calcium ($[Ca^{2+}]_m$) as a primary metabolic mediator [see Hansford (this volume) and Gunter, T. E., Gunter, K. K., Sheu, S.-S., and Gavin, C. E. (1994) *Am. J. Physiol.* **267,** C313–C339, for reviews] has emphasized the importance of understanding the characteristics of those mechanisms that control $[Ca^{2+}]_m$. In this review, we attempt to update the descriptions of the mechanisms that mediate the transport of Ca^{2+} across the mitochondrial inner membrane, emphasizing the energetics of each mechanism. New concepts within this field are reviewed and some older concepts are discussed more completely than in earlier reviews. The mathematical forms of the membrane potential dependence and concentration dependence of the uniporter are interpolated in such a way as to display the convenience of considering V_{max} to be an explicit function of the membrane potential. Recent evidence for a transient rapid conductance state of the uniporter is discussed. New evidence concerning the energetics and stoichiometries of both Na^+-dependent and Na^+-independent efflux mechanisms is reviewed. Explicit mathematical expressions are used to describe the energetics of the system and the kinetics of transport via each Ca^{2+} transport mechanism.

KEY WORDS: Mitochondria; transport; calcium; metabolic mediator; kinetics; calcium pulses.

INTRODUCTION

Mitochondrial Ca^{2+} transport was discovered in the early 1960s (DeLuca and Engstrom, 1961). Prior to the chemiosmotic revolution in mitochondrial bioenergetics, it was viewed as active uptake followed by passive release through an undefined leak (Chance, 1965). Recognition of the existence of a large, internally negative membrane potential across the inner membrane (generally between 140 and 180 mV) (Brand and Murphy, 1986; Bygrave, 1977; Hansford, 1985; Mitchell, 1966a; Senior 1988) led to the realization that uptake of Ca^{2+} across the inner membrane is energetically downhill, while efflux is energetically uphill.

Under typical conditions in a cell, the membrane potential ($\Delta\psi$) may be 160 mV internally negative, mitochondrial matrix $[Ca^{2+}]_m$ may be 200 nM, external $[Ca^{2+}]_e$ 100 nM, and the pH gradient (ΔpH) 0.5 pH units (Gunter and Pfeiffer, 1990; Gunter *et al.*, 1994; Jensen *et al.*, 1986). The difference in free energy between a mole of Ca^{2+} ions outside the mitochondria and a mole of Ca^{2+} ions in the matrix space may be calculated using the expression

$$\Delta G_{Ca} = 2F\Delta\psi + RT \ln([Ca^{2+}]_e/[Ca^{2+}]_m) \quad (1)$$

where F, R, and T are the Faraday constant, the gas constant, and the temperature in kelvins, respectively. A similar expression can also be set up for any ion. Using the "typical" values given above, the free energy difference is about 29 kJ/mole for Ca^{2+}, and about 18 kJ/mole for protons. For comparison, the free energy obtained from the hydrolysis of ATP under state 3 conditions in the matrix is about 46–47 kJ/mole. In other words, the energy necessary to transport one Ca^{2+} from the matrix space to the external space is a significant fraction (about 63% here) of the energy available from ATP hydrolysis. Likewise, the same amount of energy could be obtained from the inward movement of Ca^{2+} from the external space into the matrix space.

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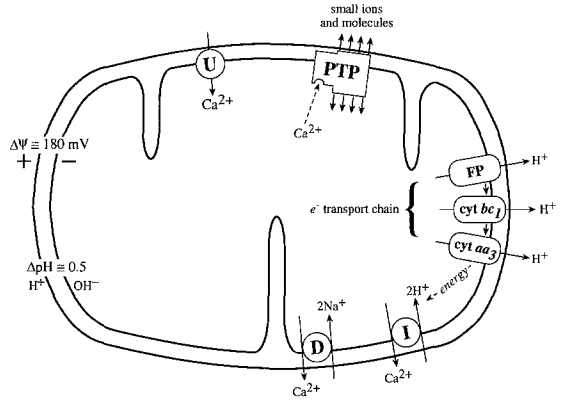

Fig. 1. Since a Ca^{2+} ion in the mitochondrial matrix is at lower energy than a Ca^{2+} ion in the external space, its influx is energetically downhill while its extrusion requires energy, as discussed in the text. The mitochondrial Ca^{2+} uniporter (U) facilitates the transport of Ca^{2+} in an inward direction down the electrochemical gradient of this ion. The Na^+-independent efflux mechanism (I) is depicted here as an active $Ca^{2+}/2H^+$ exchanger, receiving energy from the ETC (see discussion in text). The Na^+-dependent efflux mechanism (D) is depicted here as a $Ca^{2+}/2Na^+$ exchanger although, as discussed in the text, the stoichiometry may be 1 : 3. the Ca^{2+}-activated permeability transition pore is also shown for completeness although it is not discussed within the present article. It is discussed in the article by Bernardi, et al. in this volume.

There is currently evidence for three separate Ca^{2+} transport mechanisms functioning in the inner membrane of vertebrate mitochondria. Ca^{2+} influx is mediated by a uniporter while efflux is mediated by both Na^+-dependent and Na^+-independent efflux mechanisms. These mechanisms will be discussed in detail from the perspective of their energetics. In addition, the inner membrane contains a Ca^{2+}-activated, nonselective, permeability-increasing pore, which is closed in tightly coupled mitochondria. This "permeability transition pore", or PTP, sometimes categorized as a Ca^{2+} transport system, will not be discussed here but will be discussed in the accompanying article by Bernardi *et al.* Figure 1 depicts these and other relevant mechanisms within the mitochondrion.

THE UNIPORTER

General Characteristics of the Uniporter

A uniporter is a mechanism that facilitates transmembrane diffusion of an ion down its electrochemical gradient, without directly coupling that movement to that of any other ion or molecule. The mitochondrial Ca^{2+} uniporter is responsible for the rapid sequestration of external Ca^{2+} into the mitochondrial matrix, and in similar fashion also sequesters Sr^{2+}, Mn^{2+}, Ba^{2+}, Fe^{2+}, Pb^{2+}, and lanthanides in that order of selectivity (Åkerman *et al.*, 1977; Carafoli, 1965; Drahota *et al.*, 1969; Gunter and Puskin, 1972; Kapoor *et al.*, 1985; Reed and Bygrave, 1974a; Romslo and Flatmark, 1973; Vainio *et al.*, 1970). Mg^{2+} uptake is much slower than Ca^{2+} uptake and is not believed to be mediated via the Ca^{2+} uniporter (see the accompanying article by Jung and Brierley). The mitochondrial Ca^{2+} uniporter has been found in all vertebrate mitochondria tested (Bygrave, 1977; Lötscher *et al.*, 1980). Some mitochondria from invertebrates are also able to transport Ca^{2+}, but the mechanisms involved have generally not been extensively studied (Bygrave, 1977; Lötscher *et al.*, 1980).

What Is the Evidence That Mitochondria Sequester Ca^{2+} via a Uniporter?

The demonstration that the mitochondrial Ca^{2+} uptake mechanism is a uniporter took place in stages. First, it was realized that the major energy source for Ca^{2+} uptake was the internally negative membrane potential across the inner membrane. Second, a series of studies demonstrated that mitochondrial Ca^{2+} uptake was not directly coupled to transport of any other species. Finally, mitochondrial Ca^{2+} uptake was demonstrated to have the characteristics of diffusion down the Ca^{2+} electrochemical gradient.

After the introduction of chemiosmotic concepts into bioenergetics, it was recognized that a membrane potential could be generated either through substrate oxidation and electron transport (Mitchell, 1966b) or through ATP hydrolysis on the mitochondrial F_1ATPase (Scarpa and Azzone, 1970). When the membrane potential was generated by substrate oxidation, Ca^{2+} uptake was inhibited by metabolic inhibitors but not by oligomycin, which inhibits the ATPase (Lehninger *et al.*, 1967). When the membrane potential was generated by ATP hydrolysis, oligomycin would inhibit Ca^{2+} uptake but metabolic inhibitors would not (Lehninger *et al.*, 1967). Scarpa and Azzone (1970) showed that Ca^{2+} uptake could also be supported by the potential caused by efflux of K^+ via valinomycin, an ionophore functioning as a K^+ uniporter, suggesting that Ca^{2+} uptake is electrogenic and is mediated by an internally negative membrane potential.

The possibility that Ca^{2+} uptake could be mediated by other transport mechanisms was explored in

several types of experiments. During the early 1970's many laboratories measured the H^+ ions pumped outward as each pair of reducing equivalents passed through specific regions of the electron transport chain (the $H^+/2e^-$ ratio). A popular approach to determining the coupling of Ca^{2+} was to measure $Ca^{2+}/2e^-$ ratios and to compare them with $H^+/2e^-$ and $K^+/2e^-$ ratios, the latter using valinomycin. These ratios measured the number of Ca^{2+} or K^+ ions sequestered per pair of reducing equivalents traversing the electron transport chain. The results suggested that after accounting for buffering and transport of P_i, $2H^+$ ions are pumped outward for each Ca^{2+} accumulated (Brand *et al.*, 1976; Fiskum *et al.*, 1979; Vercesi *et al.*, 1978), and also that Ca^{2+} is sequestered half as fast as K^+, mole for mole (Azzone *et al.*, 1976). When Ca^{2+} accumulation is driven by K^+ efflux via valinomycin, the K^+/Ca^{2+} ratio is 2/1 (Åkerman, 1978b; Azzone *et al.*, 1976; Fiskum *et al.*, 1979). Furthermore, Ca^{2+} and Mn^{2+} uptake were shown not to be accompanied by K^+ efflux and to be unaffected by varying the concentration of K^+ (Lehninger, 1972; Puskin *et al.*, 1976). These results suggested that when a Ca^{2+} ion was sequestered via the Ca^{2+} uptake mechanism the net charge transfer was 2, as it would be for a uniporter.

In experiments of a very different type, Selwyn *et al.* (1970), using metabolically inhibited mitochondria, conducted passive swelling studies designed to demonstrate the conditions under which Ca^{2+} uptake resulted in rapid osmotic swelling. Swelling only occurred when Ca^{2+} and a charge- and pH-balancing anion were sequestered simultaneously. Thus Ca^{2+} uptake with the chaotropic (and membrane-permeable) anion SCN^- caused swelling, since SCN^- could cross the membrane directly to balance charge. However, Ca^{2+} with the anion Ac^- did not cause swelling, since acetate crosses the membrane in the HAc form, and its uptake with Ca^{2+} would cause a charge and pH imbalance. If Ca^{2+} had been accumulated via a $Ca^{2+}/2H^+$ exchanger, the charge and pH would be balanced; thus lack of swelling indicated that Ca^{2+} does not cross the membrane via a proton exchanger. Using experiments and logic of this type, Selwyn *et al.* (1970) eliminated all of the likely possibilities for the mitochondrial Ca^{2+} uptake mechanism except for a uniporter and an electrogenic Ca^{2+}/K^+ exchanger. However, the possibility of a Ca^{2+}/K^+ exchanger was ruled out by the $Ca^{2+}/2e^-$ and $K^+/2e^-$ experiments described above and by other independent studies (Lehninger, 1972; Puskin *et al.*, 1976). All of these studies showed that Ca^{2+} uptake is not directly coupled to transport of any other type of ion.

Finally, Wingrove *et al.* (1984), studying uptake of carefully buffered levels of Ca^{2+}, showed that the uptake velocity vs membrane potential dependence for the uptake mechanism fit the equations of electrochemical diffusion (discussed below). Therefore, the Ca^{2+} uptake mechanism shows all of the characteristics of a uniporter.

Kinetics of the Uniporter

Activators and Inhibitors of the Uniporter

Many types of ions and compounds act to inhibit or activate the uniporter. First, Sr^{2+} (Carafoli, 1965), Mn^{2+} (Baker and Schlaepfer, 1978), Ba^{2+} (Åkerman *et al.*, 1977), Fe^{2+} (Romslo and Flatmark, 1973), and lanthanides (Reed and Bygrave, 1974a) are similar enough to Ca^{2+} to act as competitive inhibitors. Second, Mg^{2+}, H^+ and polyamines are not thought to be transported over the uniporter, but their binding to or near to the uniporter either activates or inhibits Ca^{2+} transport. Third, polycations like Ru360 (Ying *et al.*, 1991), hexamine cobalt, or ruthenium red (Tashmukhamedov *et al.*, 1972) are among the strongest inhibitors of the uniporter but are not thought to bind to the transport site. Finally, quite a few pharmacological agents such as local anesthetics, cardioactive drugs, diuretics, and β-blockers show inhibitory effects on the uniporter. Two types of activators of the uniporter are known. First, some transported ions, especially Ca^{2+} (Vinogradov and Scarpa, 1973), and certain lanthanides (e.g., Pr^{3+}) (Vainio *et al.*, 1970), seem to function by binding to to an activator site and thereby affecting binding or transport at the transport site. Second, specific polyamines, particularly spermine, activate Ca^{2+} uptake at low $[Ca^{2+}]$ (Kröner, 1988; Lenzen *et al.*, 1992; Nicchitta and Williamson, 1984; Rottenberg and Marbach, 1990a). At higher $[Ca^{2+}]$, spermine can inhibit Ca^{2+} uptake (Lenzen *et al.*, 1992; Nicchitta and Williamson, 1984).

Ruthenium red, a hexavalent polysaccharide stain is often used to label glycoproteins, is the most frequently used inhibitor of the mitochondrial uniporter, although it also inhibits other important cellular functions, for example, Ca^{2+} flux through both the cardiac and skeletal muscle forms of the Ca^{2+} release protein of the sarcoplasmic reticulum (Meissner and Henderson, 1987). The recrystallized

form of ruthenium red can be used (Luft, 1971); however, the forms present in the crude preparation seem to inhibit influx to about the same extent. Ruthenium red inhibits the uniporter noncompetitively with a K_i of around 30 nM (Reed and Bygrave, 1974b). Because this compound binds to glassware, plastic ware, and medium components (e.g., BSA), the relationship between the total amount of ruthenium red and the level of free ruthenium red must generally be determined under experimental conditions by assaying the rate of Ca^{2+} accumulation via the uniporter. Ru360, another ruthenium-containing compound, has been shown to inhibit Ca^{2+}-stimulated respiration by 50% at 3.5 pmol/mg protein, or 17 times less than the concentration of ruthenium red which inhibits by this amount Ying *et al.*, 1991).

Mela (1969) and Reed and Bygrave (1974b) titrated uniporter activity using ruthenium red to estimate the maximum possible uniporter concentration, assuming that one ruthenium red molecule could inhibit one uniporter. Mela (1969) extrapolated directly from the initial slope of the inhibition curve, while Reed and Bygrave (1974b) made use of the mathematical theory of Henderson (1972). Mela concluded that the uniporter could be present at a concentration within the inner membrane of no higher than 0.01 nmol/mg protein (Mela, 1969), while Reed and Bygrave (1974b) obtained 0.001 nmol/mg protein as the upper limit.

For more detailed information about other uniporter inhibitors, see Gunter and Pfeiffer (1990).

Dependence of Uptake on Membrane Potential.

Wingrove *et al.* (1984) measured uptake velocity as a function of $\Delta\psi$ for three buffered levels of $[Ca^{2+}]$ (0.5, 1.0, and 1.5 μM) over the range from around 80 to 180 mV. Buffering $[Ca^{2+}]$ in this range during Ca^{2+} uptake causes the load on the proton pumping system—and therefore on $\Delta\psi$—to be approximately constant with time (see next section). Wingrove *et al.* varied the value of this "steady-state" membrane potential by adding malonate, which competes with the substrate succinate for transport and binding within the electron transport chain. Ca^{2+} uptake velocity was measured using the cold quench and rapid filtration techniques with $^{45}Ca^{2+}$. $\Delta\psi$ was measured using a calibrated tetraphenylphosphonium (TPP) electrode. The resulting plots of influx vs $\Delta\psi$ were fit with several functional forms consistent with a Nernstian distribution across a uniporter under steady-state conditions. (This type of distribution is thermodynamically required at equilibrium for transport via a uniporter.) The results (see Gunter and Pfeiffer, 1990) showed a much closer fit to the functional form

$$[e^{\Delta\phi/2}\Delta\phi/2]/[\sinh \Delta\phi/2] \quad (2)$$

than to any other functional form used. In this equation, $\Delta\phi = b(2F/RT)(\Delta\psi - \Delta\psi_0)$, F, R and T are the Faraday constant, gas constant, and temperature in kelvins, respectively, and $\Delta\psi$ is the transmembrane electrical potential. The quantities b and $\Delta\psi_0$ are fitting parameters representing the fraction of the total transmembrane potential spanned by the uniporter and any offset in zero membrane potential caused by local fixed charges, respectively. The best fit values of b and $\Delta\psi_0$ were found to be 1 and 91 mV, respectively, indicating that while transport via the uniporter spanned the entire transmembrane potential, there was an offset due to the effect of local fixed charges. This functional form [Eq. (2)] was derived using assumptions identical to those used in deriving the Goldman constant field equation (Gunter and Pfeiffer, 1990; Wyssbrod *et al.*, 1971); i.e., the electric field across the membrane is constant, the flux is independent of position across the membrane, and the ions move down their electrochemical gradient through electrochemical diffusion. Hence these results indicate that this mechanism mediates Ca^{2+} transport down the Ca^{2+} electrochemical gradient.

Concentration Dependence of Mitochondrial Uniporter Kinetics

Several difficulties are encountered in measuring accurately the velocity of Ca^{2+} uptake via the uniporter. Reviewing these difficulties before reviewing transport via this mechanism may help to convey the limits of our understanding of the uniporter. First, the uniporter is a rapid transport mechanism. In a suspension of mitochondria at 1 mg/ml, it can deplete the free Ca^{2+} in a suspension of 10–50 μM in seconds. Consequently, accurate measurement of Ca^{2+} uptake at a given $[Ca^{2+}]$ requires that the $[Ca^{2+}]$ be strongly buffered. Second, the uniporter is electrophoretic: Ca^{2+} uptake decreases the membrane potential. Rapid uptake via the uniporter can overwhelm the ability of proton pumping by the electron transport chain to rebuild the membrane potential, thus causing a larger decrease in $\Delta\psi$ as influx increases. Finally, because of the great difference in

transport velocity observed between the low $[Ca^{2+}]$ (i.e., physiological) range and the range above about 5 μM (i.e., pathological range), the same techniques for measurement of influx cannot be used with accuracy over the full range (Sparagna *et al.*, 1994; Wingrove and Gunter, 1986a,b).

While the first problem mentioned above can be solved in the low $[Ca^{2+}]$ region of interest by use of Ca^{2+} buffers, the second requires considerably more work. Optimum conditions for measurement of Ca^{2+} uptake velocity would include constancy of $\Delta\psi$; however, we cannot control the system sufficiently to maintain this condition. The best that we can do is to work over a range of measured $\Delta\psi$. Variation of $\Delta\psi$ can be achieved in a variety of ways, the simplest being addition of varying amounts of a metabolic inhibitor to the suspending medium. Then, as described above, transport velocity can be plotted vs $\Delta\psi$ over a range of buffered $[Ca^{2+}]$'s, curves can be fit to the data at each $[Ca^{2+}]$, and the values of transport velocity at a given $\Delta\psi$ taken from these plots for each value of $[Ca^{2+}]$. A plot of transport velocity vs $[Ca^{2+}]$ at constant $\Delta\psi$ can thus be obtained in order to determine the concentration dependence of transport. Unfortunately, because rapid Ca^{2+} uptake severely "loads" the system for regenerating $\Delta\psi$, there is currently no effective way to control either $[Ca^{2+}]$ or $\Delta\psi$ during Ca^{2+} uptake near V_{max}. The measured values of V_{max} for this mechanism may not be reliable, then, because they are compromised by measurement at decreased $\Delta\psi$.

Bygrave *et al.* (1971) first measured Ca^{2+} uptake velocity into isolated liver mitochondria using the ruthenium red quench technique. Soon after, Scarpa and Graziotti (1973) used the metallochromic indicator, murexide, to obtain similar data with heart mitochondria. Both of these studies showed sigmoidicity in the plot of Ca^{2+} uptake velocity vs $[Ca^{2+}]$, indicating higher-order transport kinetics. Subsequent studies showed a large variation in transport parameters, due both to varying experimental conditions and to the effects of the problems discussed above. For example, the observed V_{max} varied between 700 and 1200 nmol/mg · min for liver mitochondria (Bragadin *et al.*, 1979a, Hutson *et al.*, 1976; Vinogradov and Scarpa, 1973), between 280 and 600 nmol/mg · min for heart mitochondria prepared by mechanical tissue disruption (Crompton *et al.*, 1976b; McMillin-Wood *et al.*, 1980), and between 400 and 1750 nmol/mg · min for heart mitochondria prepared by the nagarse technique (McMillin-Wood *et al.*, 1980; Vercesi *et al.*, 1978). These results also generally showed sigmoidicity.

The order of a transport process is usually determined by plotting the transport velocity vs concentration data in a Hill plot. This plot linearizes the data from simple transport of nth order (i.e., linearizes the nth-order Hill equation) through use of the form

$$\log \{v/(V_{max}^{app} - v)\} = n \log [Ca^{2+}] - n \log K_{0.5} \quad (3)$$

In the Hill plot, $\log \{v/(V_{max}^{app} - v)\}$ is plotted against $\log [Ca^{2+}]$ so that the slope yields n, the Hill coefficient. Note that the apparent V_{max}, V_{max}^{app}, is looked upon as a function of membrane potential [or $V_{max}^{app}(\Delta\psi)$] and corresponds to the value of maximum velocity at the membrane potential of interest. V_{max}^{app} will be related below to the membrane potential independent value, $\bar{v}_{max}$. Most studies of the uniporter under a wide variety of conditions have shown sigmoidicity in the velocity vs $[Ca^{2+}]$ dependence, yielding a Hill coefficient greater than one and indicating some form of positive cooperativity (Bragadin *et al.*,1979a; Bygrave *et al.*, 1971; Heaton and Nicholls, 1976; Hutson *et al.*, 1976; Reed and Bygrave, 1975; Scarpa and Graziotti, 1973; Vinogradov and Scarpa, 1973). The degree of this cooperativity has varied with conditions, and particularly with ionic strength, temperature and $[Mg^{2+}]$ (Åkerman, 1977a,b; Bragadin *et al.*, 1979b; Crompton *et al.*, 1976b; Kröner, 1986b). Generally the Hill coefficient has been found to lie between about 1.7 and 2.0, although a wider range of values has been observed.

As an example of measurement of Hill coefficients, the transport data of Wingrove *et al.* (1984) and of Gunter and Pfeiffer (1990) are plotted in Fig. 2. These data were taken to analyze the membrane potential dependence of the uniporter, not the concentration dependence. Consequently, emphasis was placed on strongly buffering $[Ca^{2+}]$ at each of three values of $[Ca^{2+}]$ but not on accurate determination of $[Ca^{2+}]$. However, since this represents the only extensive set of data for which membrane potential corrections have been made, it must suffice for the current example. Figure 3A of Gunter and Pfeiffer (1990) shows uniporter uptake velocity as a function of membrane potential at three buffered concentrations of $[Ca^{2+}]$, and the functional form representing the membrane potential dependence is fit to the data at each concentration. In Fig. 2, Hill plots are made from these data. The maximum observed transport velocity in mitochondria similar to the preparations used

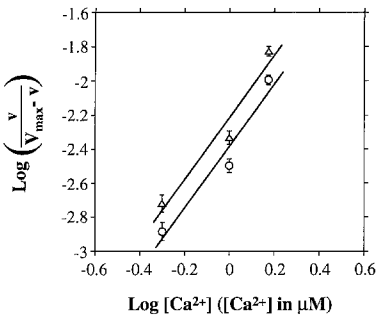

Fig. 2. Hill plot of Ca^{2+} uniporter data corrected for changes of membrane potential. Each point shown here represents all of the data points (14–15) obtained by Wingrove *et al.* (1984) for a given $[Ca^{2+}]$ and shown fit to the "Goldman" functional form in Fig. 3A of Gunter and Pfeiffer (1990). Therefore each data point shown here represents 14 or 15 measurements taken at transmembrane potentials between −87.1 and −186.6 mV. Each of the 14 or 15 values represented by each symbol on the plot represents the coefficient of the fit to the functional form $[e^{\Delta\phi/2}\Delta\phi/2]/[\sinh \Delta\phi/2]$ which corrects for the variation due to membrane potential. For the data indicated by triangles, V_{max}^{app} was assumed to be 1200 nmol/mg · min at 110 mV, while for the data indicated by circles V_{max}^{app} at that membrane potential was assumed to be 1750 nmol/mg · min. The limits represent one standard deviation from the average, and each of the lines represents the best fit to one of the two sets of data. The average value of the slopes of these lines and hence the measured Hill coefficient is 1.956, while the intercepts of these lines of best fit with the vertical line at $[Ca^{2+}] = 1\ \mu M$, yields 13.4 and 16.4 μM, respectively for the value of $K_{0.5}$.

by Wingrove *et al.* (1984) has been above 1200 nmol/mg · min at a membrane potential below about 110 mV, as determined by a calibrated tetraphenylphosphonium (TPP) electrode (Jensen *et al.*, 1986). In Fig. 2 we have taken this into account by considering values of V_{max}^{app} in the range of 1200–1750 nmol/mg · min at 110 V and equivalent values of V_{max}^{app} (i.e., corrected for $\Delta\psi$ differences through the known membrane potential dependence of transport via this mechanism, as described above).

While the resultant Hill plots suffer from containing only three points corresponding to the three concentrations studied (Wingrove *et al.*, 1984) and from suboptimal accuracy of $[Ca^{2+}]$, they still serve to demonstrate how to determine important parameters describing the concentration dependence of transport via the uniporter. First, although there are only three Ca^{2+} concentrations, the data points at each concentration are the means of the 14 or 15 independent measurements made at membrane potentials ranging from −87.1 mV to −186.6 mV. The small standard deviations in the data over this range of $\Delta\psi$ indicate the good internal consistency of the $\Delta\psi$ fit (Gunter and Pfeiffer, 1990). Surprisingly, our lack of precise knowledge of $\bar{v}_{max}$ (or V_{max}^{app}) is not a serious limitation to our ability to determine $K_{0.5}$ and the Hill coefficient n from these data. The major limitations are the small number of points on each plot and the error in $[Ca^{2+}]$. These results support a value of $n = 1.956 \pm 0.016$ and a value of $K_{0.5}$ in the range of 13.4–16.4 μM. Even though we varied the value of V_{max}^{app} by 45%, the resultant change in $K_{0.5}$ was only 22%.

Consistent with these results, Vinogradov and Scarpa (1973) found that small amounts of Ca^{2+} or Pr^{3+} cause loss of the sigmoidicity of Mn^{2+} uptake via the uniporter. The interpretation is that the uniporter possesses both an activation site and a transport site (Kröner, 1986a,b; Vinogradov and Scarpa, 1973). Binding of Ca^{2+} or Pr^{3+} to the activation site either lowers the activation energy required for transport or lowers the K_d for binding to the transport site. This is consistent with studies of the revese activity of the uniporter, where it has been observed that the chelator EGTA is almost as effective as ruthenium red in inhibiting uncoupler-induced Ca^{2+} release (Igbavboa and Pfeiffer, 1988, 1991a,b; Leikin and Gonsalves, 1986). EGTA is viewed as preventing Ca^{2+} binding to the activation site by keeping the $[Ca^{2+}]$ significantly below the K_d and thereby making efflux via the uniporter very slow.

Published values of $K_{0.5}$ have ranged from around 1 μM to 189 μM, with the value of $K_{0.5}$ correlating with the estimated value of V_{max} (Åkerman, 1977a,b; Crompton *et al.*, 1976b; Hutson, 1976, 1977; Pfeiffer *et al.*, 1976; Reed and Bygrave, 1975; Vinogradov and Scarpa, 1973). The best consensus value is 10–20 μM (consistent with the results of Fig. 2). While agreement with respect to transport parameters is poor, the best interpretation of the concentration dependence data is that the transport velocity fits the functional form

$$v = V_{max}^{app}[Ca^{2+}]^2/(K_{0.5}^2 + [Ca^{2+}]^2) \qquad (4)$$

where $V_{max}^{app} = \bar{v}_{max}[e^{\Delta\phi/2}\Delta\phi/2]/[\sinh \Delta\phi/2]$, and $\Delta\phi$ is defined in the section on the membrane potential dependence of the uniporter, above. The best value of the membrane potential-independent parameter $\bar{v}_{max}$ for the Ca^{2+} uniporter in liver mitochondria is in the range of 600–900 nmol/mg · min.

Activation Energies and the Temperature Dependence of Uptake

Transport processes consist of a series of steps: binding of the substrate to the transport site on the

"cis" side of the membrane, translocation (which often involves a conformational change in the transport complex), and release of the substrate from the "trans" side of the membrane. If translocation is rate limiting, as it often is, then the temperature dependence of the transport rate is a measure of the activation energy of translocation by plotting the transport rate data as a function of the kelvin temperature in an Arrhenius plot [log(rate) vs $1/T$]. The slope of this plot yields the activation energy.

Without proof that the translocation step is rate limiting, such "activation energies" may not be relevant to the transport process; however, there is no harm in reviewing the data. Åkerman (1977b) measured an "activation energy" of around 40 kJ/mole for Ca^{2+} uptake via the uniporter with mitochondria suspended in sucrose medium and energized by succinate oxidation. Addition of 2 mM Mg^{2+} lowered the activation energy to 18 kJ/mole and addition of 0.4 mM spermine lowered the activation energy to approximately zero (Åkerman, 1977b). Mg^{2+} inhibits Ca^{2+} influx; however, there is reason to believe that it can bind to the activation site. Mg^{2+} or spermine binding to the activation site may lower the "activation energy", while Mg^{2+} inhibition of Ca^{2+} transport could relate to competition between these ions for binding to the transport site. A lanthanide that competitively inhibits Ca^{2+} transport showed no effect on the activation energy, while suspension of the mitochondria in KCl medium as opposed to sucrose medium increased the "activation energy" to around 70 kJ/mole (Åkerman, 1977b). The possibility that the rate-limiting step is not translocation but rather the rebuilding of the membrane potential through proton pumping, creates a serious caveat to the interpretation and relevance of this type of data. To address this question, Bragadin *et al.* (1979a,b) used K^+ efflux via valinomycin instead of substrate oxidation to rebuild the membrane potential. The "activation energy" measured in this way in sucrose medium (41 kJ/mole) was in good agreement with that measured in similar medium using succinate oxidation, suggesting that what is being measured is the activation energy of the translocation step.

Reversibility of the Uniporter

Thermodynamics requires that a passive mechanism be reversible, subject to its driving forces. Earlier work suggested that the uniporter is reversible (see discussion in Gunter and Pfeiffer, 1990). However, Kapùs *et al.* (1991) have reported that the uniporter does not sequester Ca^{2+} in the absence of a membrane potential, even in the presence of an 8-fold [Ca^{2+}] gradient. They interpret this to mean that the membrane potential holds the uniporter in a transport-competent conformation. It is certainly possible that the membrane potential influences the conductivity of the uniporter through control of molecular conformation. However, rapid loss of cations through the uniporter following a loss of membrane potential has often been reported (Mela, 1969; Puskin *et al.*, 1976; Vasington *et al.*, 1972; Wingrove and Gunter, 1986b). These new observations, then, can be interpreted as arguing against complete uniporter reversibility. Accurate measurement of membrane potential is very difficult below around 80–100 mV. Perhaps during release of cations through the uniporter, the electrogenic cation release itself increases the internally negative membrane potential sufficiently to maintain the transport-competent conformation of the uniporter and cause the observed distinction between Ca^{2+} release via the uniporter and the conditions used by Kapus *et al.* (1991).

Interaction of the Uniporter with Pulses of Ca^{2+}

The Characteristics of the [Ca^{2+}] to Which Mitochondria Are Exposed In Vivo

Generally, the uniporter has been studied under conditions either of steady-state (buffered) [Ca^{2+}] or of [Ca^{2+}] that was not controlled and therefore varied with experimental conditions. The question of what [Ca^{2+}] the mitochondria are exposed to in the cell is central to solving the riddle of the physiological role of mitochondrial Ca^{2+} transport. As our understanding of intracellular [Ca^{2+}] has increased by use of the bioluminescent compound aequorin and of the ratiometric fluorescent indicators fura-2 and indo-1, the answer to this question has become obtainable (Cobbold and Rink, 1987).

Under pathological conditions in which the integrity of the plasma membrane has been either physically or chemically breached, changing its permeability to Ca^{2+}, or in which cytosolic ATP levels become depressed (e.g., ischemia), steady-state $[Ca^{2+}]_c$ in some tissues can rise significantly. In many cases, changes in $[Ca^{2+}]_c$ take place slowly and our earlier understanding of uniporter kinetics is probably sufficient to explain most observations.

Ca^{2+} is often readily accumulated by mitochondria under pathological conditions, and mitochondrial Ca^{2+} overload is observed in over 95% of cell death (Farber, 1981).

Under physiological conditions, $[Ca^{2+}]_c$ is generally low: near 100 nM (Fiskum, 1985) or lower (Gunter *et al.*, 1990) unless the cell has been excited either through neuronal stimulation or by a hormone or growth factor. Following stimulation, pulses of Ca^{2+} and sometimes sequences of pulses are often observed (Berridge and Galione, 1988; Berridge and Moreton, 1991; Berridge, 1993; Gilon *et al.*, 1993; Petersen *et al.*, 1993; Sheu and Blaustein, 1992). While the pulse characteristics vary with cell type and agonist, the strongest pulses are often as high as 1–2 μM, the duration from 0.5 sec or less in heart to as long as a minute in metabolically inactive tissue such as chondrocytes (Zuscik, 1993), and the periodicity (if more than one pulse is observed) may vary from less than a second to several minutes (Cobbold and Rink, 1987).

Sequestration of Ca^{2+} Pulses by Mitochondria

Since Ca^{2+} uptake is second order and the rate of sequestration following activation is almost nine times that prior to activation, the amount of uptake from a single pulse should be very dependent on the level of uniporter activation and on the history of $[Ca^{2+}]_c$ in the vicinity of the mitochondria. It is impossible to predict from steady-state data what the uptake would be from a cytosolic Ca^{2+} pulse or pulse sequence. Therefore, the earlier data on mitochondrial Ca^{2+} uptake kinetics are not very useful in predicting mitochondrial Ca^{2+} uptake and intramitochondrial $[Ca^{2+}]_m$ following exposure of the mitochondria to a Ca^{2+} pulse or pulse sequence. What is needed are direct studies of mitochondrial Ca^{2+} uptake from pulses. Only two relevant studies have been published.

Leisey *et al.* (1993) bound heart mitochondria to a glass sample tube, passed a stream of medium over them in which the $[Ca^{2+}]$ varied periodically (almost sinusoidally), and observed changes in $[Ca^{2+}]_m$ using the ratiometric Ca^{2+} indicator fura-2. While sensitivity did not permit an increase in $[Ca^{2+}]_m$ to be observed following a single pulse, an increase was observed following a sequence of pulses. The half time for an increase in $[Ca^{2+}]_m$ to a new steady state following an increase in the average $[Ca^{2+}]$ of the medium was found to be slow (about 2 min at 22°C).

Sparagna *et al.* (1994), using liver mitochondria and a very different experimental design, were able to observe increases in total Ca^{2+} following exposure of the mitochondria to one or more pulses fashioned either as square waves or similar to those observed in the cytosol of hepatocytes after activation by hormones. These experiments were performed on suspensions of mitochondria in cyclindrical fluorescence cuvettes. Medium $[Ca^{2+}]$ was determined by a computer-controlled, dual pipetter system that could be programmed to produce the desired sequence of $[Ca^{2+}]$. To do this, either buffered Ca^{2+} or Ca^{2+} buffer was rapidly injected into the cuvette, where it was rapidly mixed by stirring. The complete rise or fall times for $[Ca^{2+}]$ under experimental conditions took about 0.4 s on average, but could be shorter than 0.25 s when necessary. Ca^{2+} uptake was measured using $^{45}Ca^{2+}$ and dual label techniques. By using fura-2-loaded-mitochondria, $[Ca^{2+}]_m$ could also be measured following external $[Ca^{2+}]$ pulses when desired. This system has the advantages of high reproducibility and accuracy of measurement.

The results of these studies indicate very rapid Ca^{2+} uptake into mitochondria even at quite low

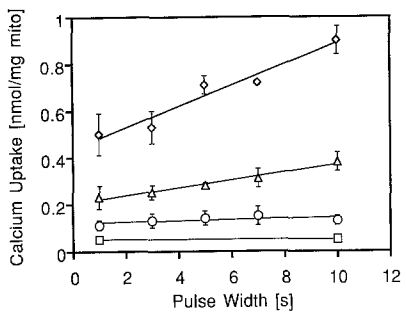

Fig. 3. Mitochondrial calcium uptake from pulses. Mitochondria were exposed to $^{45}Ca^{2+}$ pulses of various widths between 1 and 10 s, and the resulting amount of $^{45}Ca^{2+}$ uptake by the mitochondria was determined using counting techniques as described by Sparagna *et al.* (1994). The pulses were made by injecting 2.7 mM $^{45}Ca^{2+}$ buffered by 18 mM HEDTA followed at the end of the desired pulse duration by injecting 2.5 mM EGTA. The data indicated by diamonds represent pulses with an average height of 566 nM $[Ca^{2+}]$, as detemined by the Ca^{2+}-indicator fura-2. The data indicated by triangles represent pulses with an average height of 364 nM. The data indicated by circles represent pulses with an aveage height of 199 nM. In the control data indicated by squares, 4 nmol of ruthenium red per mg protein was pippetted into the cuvette by hand prior to injection of HEDTA-buffered Ca^{2+} followed by injection of EGTA, forming pulses of average height 436 nM $[Ca^{2+}]$. Error bars represent one standard deviation of three repetitions. As can be seen in this figure, the period of very rapid uptake rate is less than 1 s. This period of very rapid uptake is followed by an extended period of slower uptake. Uptake of both of these types is inhibited by ruthenium red.

values of [Ca^{2+}] (200–500 nM) (see Fig. 3). Control experiments showed that this was net uptake as opposed to either binding or exchange of labeled external Ca^{2+} for cold internal Ca^{2+}. The interesting thing about these results is that they suggest that the uniporter has at least two conductivity states independent of Ca^{2+} binding to the activation site. At very short times (less than 1 s), uptake is very fast, then it slows down at times greater than 1 s. This slower uptake is consistent with earlier steady-state results (Sparagna *et al.*, 1994). This suggests that the uniporter has special properties to facilitate the uptake of Ca^{2+} from short pulses and that it can also sequester large amounts of Ca^{2+} in a slower mode under longer-term exposure to Ca^{2+}. These properties have not been recognized earlier because their detection requires accurate measurements of uptake at very short times (pulse duration) and at low values of [Ca^{2+}].

THE CA^{2+} EFFLUX MECHANISMS

Two separate Ca^{2+} efflux mechanisms, the Na^+-dependent (Crompton *et al.*, 1976a) and the Na^+-independent (Puskin *et al.*, 1976) mechanisms, have been identified in vertebrate mitochondria. Usually the Na^{2+}-dependent mechanism is thought of as dominating the Ca^{2+} efflux of mitochondria from heart, brain, skeletal muscle, parotid gland, adrenal cortex, brown fat, and other excitable cells (Crompton *et al.*, 1977, 1978; Murphy and Fiskum, 1988), while the Na^+-independent mechanism is considered dominant in mitochondria from liver, kidney, lung, and smooth muscle (Crompton *et al.*, 1978; Fiskum and Lehninger, 1979; Puskin *et al.*, 1976). There is evidence that Na^+-dependent efflux exists in liver mitochondria (Goldstone and Crompton, 1982; Heffron and Harris, 1981; Wingrove and Gunter, 1986b) and Na^+-independent efflux in heart mitochondria (Crompton *et al.*, 1977; Rosier *et al.*, 1981). In fact, the Na^+-independent mechanism in liver has a lower V_{max} than the Na^+-dependent mechanism in the same tissue; however, in liver the Na^+-independent mechanism is much less sensitive to inhibition by physiological [Mg^{2+}]$_c$ than is the Na^+-dependent mechanism (Gunter and Pfeiffer, 1990; Wingrove and Gunter, 1986a,b).

The Na^+-Independent Ca^{2+} Efflux Mechanism

It has been suggested by prominent authors in mitochondrial Ca^{2+} transport that what is called the Na^+–independent Ca^{2+} efflux mechanism may not exist, but that its manifestations are possible artifacts either of reversed uniport, the mitochondrial membrane permeability transition, or Na^+-dependent Ca^{2+} efflux (Crompton, 1990; McCormack and Denton, 1993; Pfeiffer *et al.*, 1983). [The discussion in the review by Crompton (1990) is especially clear in this regard.] Until recently, this was an objective and defensible position. However, the existence of a separate Na^+-independent efflux mechanism transporting Ca^{2+} in parallel with the Na^+-dependent mechanism is clearly supported by the following evidence: (1) persistent observations of reproducible Ca^{2+} efflux (especially from liver mitochondria) under energized conditions in the absence of Na^+ (Bernardi and Azzone, 1979; Gunter *et al.*, 1983,1991; Gunter and Pfeiffer, 1990), in the presence of inhibitors of Na^+-dependent efflux (Gunter and Pfeiffer, 1990; Gunter *et al.*, 1991; Wingrove and Gunter, 1986a), and in the presence of inhibitors of the permeability transition (Gunter *et al.*, 1991); (2) evidence that this Na^+-independent efflux is inhibited by other agents than those that inhibit Na^+-dependent efflux (see Gunter and Pfeiffer, 1990, and Gunter *et al.*, 1994 for review), (3) evidence that the kinetics of Na^+-independent efflux are well defined and are distinct from those of Na^+-dependent efflux (Gavin *et al.*, 1990; Wingrove and Gunter, 1986a,b); (4) evidence that the ion selectivity of these Ca^{2+} efflux mechanisms is distinct (Gavin *et al.*, 1990, see Gunter *et al.*, 1994 for review); and (5) evidence for outward pumping of Ca^{2+} from liver mitochondria against the Ca^{2+} electrochemical gradient, under conditions in which the uniporter, the Na^+-dependent efflux mechanism, and the permeability transition were simultaneously inhibited (Gunter *et al.*, 1991). To suggest today that the Na^+-independent Ca^{2+} efflux mechanism may not exist goes against the preponderance of the evidence.

The Na^+-independent mechanism transports Sr^{2+} (Gunter *et al.*, 1988), Ba^{2+} (Lukács and Fonyó, 1985), and Mn^{2+} (Gavin *et al.*, 1990, 1991; Gunter *et al.*, 1978b; Puskin *et al.*, 1976) as well as Ca^{2+}. It is inhibited by Sr^{2+} (Gunter *et al.*, 1988; Saris and Bernardi, 1983), Mn^{2+} (Gavin *et al.*, 1990,1991), by low levels of the classical uncouplers FCCP, CCCP, and DNP (Bernardi and Azzone, 1982; Gunter *et al.*, 1978b, 1979; Ligeti and Lukács, 1984; Rosier *et al.*, 1981), by very high levels of the lipophilic cations tetraphenylphosphonium (TPP) (Gunter *et al.*, 1988; Wingrove and Gunter, 1986b) and triphenylmethyl-

phosphonium (TPMP) (Wingrove and Gunter, 1986b), by CN (Gunter and Pfeiffer, 1990; Gunter and Gunter, 1985; Gunter *et al.*, 1987b), and by very high levels of ruthenium red (Wingrove and Gunter, 1986b). Mn^{2+} inhibition of this mechanism is competitive (Gavin *et al.*, 1990). Kinetically, Na^+-independent efflux is second order and is a non-essential activation mechanism (Gavin *et al.*, 1990; Gunter and Pfeiffer, 1990; Gunter *et al.*, 1988; Wingrove and Gunter, 1986a). Transport of Ca^{2+} via this mechanism in liver fits the kinetic form

$$v = \frac{V_{max}\{([Ca^{2+}]^2 + a[Ca^{2+}])}{(K_m^2 + [Ca^{2+}]^2 + 2a[Ca^{2+}])\}}, \quad (5)$$

where V_{max}, K_m, and a are fitting parameters (Wingrove and Gunter, 1986a). The best-fit values of these parameters in liver mitochondria were found to be $V_{max} = 1.2 \pm 0.1$ nmol/mg · min, $K_m = 8.4 \pm 0.6$ nmol/mg, and $a = 0.9 \pm 0.2$ nmol/mg (Wingrove and Gunter, 1986a).

When Puskin *et al.* (1976) first showed the existence of this mechanism, they observed that it could be either a passive exchanger of Ca^{2+} for another type of cation, a co-transporter of Ca^{2+} with an anion or anions, or an active mechanism. Since the Na^+-dependent mechanism has been identified as a separate mechanism above, the discussion of transporter energetics suggests that the most likely types of passive mechanism are an exchanger of Ca^{2+} for H^+ ions or a co-transporter of Ca^{2+} with P_i. The possibility that this mechanism could be a Ca^{2+}–P_i co-transporter has been ruled out by the work of Zoccarato and Nicholls (1981, 1982) and by the work of Gunter *et al.* (1991), discussed below. A number of authors have implicitly assumed that this mechanism is a passive exchanger of Ca^{2+} for H^+ without explicitly describing the mechanism as a passive exchanger (Åkerman, 1978a; Brand, 1985a; Fiskum and Cockrell, 1978; Fiskum and Lehninger, 1979; Rottenberg and Marbach, 1990b). Considerable evidence contrary to the concept of a passive $Ca^{2+}/2H^+$ exchanger has been published (Antonio *et al.*, 1991; Beatrice *et al.*, 1982; Bernardi and Azzone, 1979; Gunter *et al.*, 1983, 1991; Jurkowitz *et al.*, 1983a; Saris, 1987). A passive mechanism is defined here as one for which energy available to the mechanism at the site of transport is restricted to that from the electrochemical gradients of the transported ions. An active mechanism, then, is one using at least some energy from a source other than that of the electrochemical gradients of the transported ions. For a detailed discussion of the pros and cons of the earlier work in this area see Gunter and Pfeiffer (1990). From this work two important conclusions still appear valid. First, several approaches have suggested that this mechanism is nonelectrogenic (Brand, 1985a; Fiskum and Lehninger, 1979; Gunter *et al.*, 1983), and there is no real evidence to the contrary. Second, the energy used has been carefully investigated in an effort to determine how much energy is available to this mechanism for use in transport (Gunter *et al.*, 1991). The importance of this approach is that the maximum energy available through exchange with 2 H^+ ions can be experimentally determined. If convincing evidence can be found showing that more energy is available to this mechanism than is available from two protons passing down their electrochemical gradient, then it is impossible for this mechanism to be a simple passive exchanger of Ca^{2+} for 2 protons.

If the Na^+-independent Ca^{2+} efflux mechanism is a passive $Ca^{2+}/2H^+$ exchanger, then it should be able to transport Ca^{2+} outward in exchange for transporting 2 protons inward until

$$\{Ca^{2+}\}_e/\{Ca^{2+}\}_m = (\{H^+\}_e/\{H^+\}_m)^2 \quad (6)$$

where the curly brackets indicate activity and the subscripts e and m refer to external and mitochondrial matrix, respectively. At this point, referred to as the "null point", the energy needed to transport Ca^{2+} outward exactly balances that which can be obtained by transporting two protons inward. This result can also be expressed as

$$[Ca^{2+}]_e = [Ca^{2+}]_m(\gamma_m/\gamma_e)10^{2\Delta pH} \quad (7)$$

where γ_m/γ_e is the ratio of the Ca^{2+} activity coefficient in the mitochondrial matrix to that in the suspending medium (Gunter *et al.*, 1991). This ratio of activity coefficients was estimated using extended Debye–Huckel theory and the Davis approximation. The other necessary parameters were determined by measuring efflux and external $[Ca^{2+}]$ using the arsenazo technique, measuring mitochondrial pH gradient (ΔpH) and volume using isotope techniques, and measuring intramitochondrial $[Ca^{2+}]_m$ using the fura-2 technique (Gunter *et al.*, 1991). If the experimentally determined value of the null point for efflux of Ca^{2+} over the Na^+-independent mechanism is greater than the null point calculated using Eq. (7) above, which assumes that the mechanism is a passive $Ca^{2+}/2H^+$ exchanger, then the energy available to transport Ca^{2+} outward against its

Table I. Comparison of measured Ca^{2+} efflux null point and the null point predicted for a passive $Ca^{2+}/2H^+$ exchanger[a]

Conditions	Ca^{2+} efflux null point (μM)	$[Ca^{2+}]_i$ (nM)	ΔpH	$[Ca^{2+}]_i \frac{\gamma_i}{\gamma_0} 10^{2\Delta pH}$ (μM)	$\frac{[Ca^{2+}]_0^{meas}\gamma_0}{[Ca^{2+}]_i\gamma_i 10^{2\Delta pH}}$
Trace Ac^-	66.0	103	1.270	19.32	3.4
0.1 mM Ac^-	50.0	257	0.805	5.68	8.8
0.5 mM Ac^-	71.8	339	0.450	1.52	47.2

[a] Shown are values of the Ca^{2+} efflux null point measured as described in Gunter *et al.* (1991) in medium C (trace Ac^-), medium B (0.1 mM Ac^-), and medium A (0.5 mM Ac^-). Also shown are values of intramitochondrial $[Ca^{2+}]$ measured by the fluorescence spectroscopic analysis technique (Gunter *et al.*, 1988, 1990) as described in Gunter *et al.* (1991) and pH gradients measured by dual label techniques also as described in Gunter *et al.* (1991). Values of the Ca^{2+} efflux null point which should obtain for a passive $Ca^{2+}/2H^+$ exchanger were calculated by using these measured values and $[Ca^{2+}]_o = ([Ca^{2+}]_i(\gamma_i/\gamma_o)\, 10^{2\Delta pH})$. In all cases, the results have indicated transport against a Ca^{2+} gradient higher than that which would be possible for a passive $Ca^{2+}/2H^+$ exchanger. In the last column, the external $[Ca^{2+}]$ of the measured Ca^{2+} efflux null point in each case is divided by that value calculated using Eq. (7) for a passive $Ca^{2+}/2H^+$ exchanger.

electrochemical gradient is greater than that available to a passive $Ca^{2+}/2H^+$ exchanger. As seen from the data of Table 1 for the most extreme case studied, the experimentally observed null point is almost 50 times greater than that predicted from the assumption that the transporter is a passive $Ca^{2+}/2H^+$ exchanger. This implies that the transporter has at least 9.5 kJ/mol more energy available to it, under these conditions, than a passive $Ca^{2+}/2H^+$ exchanger would have (Gunter *et al.*, 1991). The differences are far too large to be accounted for by reasonable errors of measurement. Therefore, we must conclude that the mitochondrial Na^+-independent Ca^{2+} efflux mechanism is not a passive $Ca^{2+}/2H^+$ exchanger (Gunter *et al.*, 1991).

This is consistent with the conclusion of Rosier *et al.* (1981) who attempted to partially reconstitute this Na^+-independent mechanism. Several groups had found that low levels of uncouplers inhibited Na^+-independent Ca^{2+} efflux (Bernardi and Azzone, 1982; Gunter *et al.*, 1978b; Ligeti and Lukács, 1984; see also the discussion in Gunter and Pfeiffer, 1990). It was also found that low levels of uncoupler strongly inhibited Na^+-independent Ca^{2+} influx into submitochondrial particles energized to be internally positive (Gunter *et al.*, 1978a). Rosier *et al.* (1981) reasoned that this might be because the Na^+-independent Ca^{2+} efflux mechanism was located topographically near the high-affinity uncoupler binding site found in fraction V in mitocondria (Hatefi *et al.*, 1974). Therefore, they co-reconstituted this fraction along with cytochrome oxidase into asolectin vesicles (fraction V COV's). Studies on these vesicles showed that Ca^{2+} could be taken up against its electrochemical gradient into fraction V COV's energized to be internally positive. This could only be done when the vesicles were energized by substrate (ascorbate) oxidation. An induced, internally acid pH gradient did not mediate Ca^{2+} uptake into these vesicles. Furthermore, any inhibitor which interfered with energization of these vesicles through substrate oxidation also inhibited Ca^{2+} uptake. This Ca^{2+} uptake was also inhibited by low levels of uncouplers, similarly to the effect of uncouplers on Na^+-independent Ca^{2+} efflux from intact mitochondria. The authors concluded that this Ca^{2+} uptake was probably mediated by an active mechanism receiving energy from substrate oxidation (Rosier *et al.*, 1981). These results and those reported above suggest that the most likely identity of the Na^+-independent Ca^{2+} efflux mechanism is an active $Ca^{2+}/2H^+$ exchanger receiving at least some of its energy from the electron transport chain (Gunter *et al.*, 1991). It has been argued that such a mechanism could provide advantages in prevention of the mitochondrial membrane permeability transition under some hypoxic conditions (Gunter *et al.*, 1994).

The Na^+-Dependent Ca^{2+} Efflux Mechanism

The Na^+-dependent mechanism transports Sr^{2+} (Crompton *et al.*, 1976a) but not Mn^{2+} (Gavin *et al.*, 1990, 1991) as a substitute for Ca^{2+}; Li^+ can substitute for Na^+ (Crompton *et al.*, 1976a). Transport via this mechanism can be inhibited by external Ca^{2+} in heart (Hayat and Crompton, 1987) and activated by external Na^+ (Crompton *et al.*, 1980). Ba^{2+} inhibits this mechanism from the external side in heart mitochondria (Lukács and Fonyó, 1986) but from the internal side in liver mitochondria (Lukács and Fonyó, 1985). While earlier work, using a variety of

techniques, has supported the conclusion that this mechanism is a passive $Ca^{2+}/2Na^{+}$ exchanger, this has recently been challenged by the work of Baysal *et al.* (1994), as discussed below. The Na^{+}-dependent Ca^{2+}efflux mechanism is inhibited by a wide variety of inhibitors including Mg^{2+} (Wingrove and Gunter, 1986b), Mn^{2+} (Gavin *et al.*, 1990, 1991), external Ca^{2+} (Hayat and Crompton, 1987), trifluoperazine (Hayat and Crompton, 1985), diltiazem (Chiesi *et al.*, 1987; Rizzuto *et al.*, 1987), verapamil (Wolkowicz *et al.*, 1983), clonazepam (Chiesi *et al.*, 1987), bepridil (Chiesi *et al.*, 1987), amiloride (Jurkowitz *et al.*, 1983a,b; Schellenberg *et al.*, 1985; Sordahl *et al.*, 1984), tetraphenylphosphonium (Wingrove and Gunter, 1986b), triphenylmethylphosphonium (Wingrove and Gunter, 1986b), and high levels of ruthenium red (Wingrove and Gunter, 1986b). Transport via this mechanism is activated by spermine and spermidine (Nicchitta and Williamson, 1984) and stimulated in brain mitochondria by ethanol (Rottenberg and Marbach, 1991). It has been described both in heart (Crompton *et al.*, 1977) and in liver (Wingrove and Gunter, 1986b) as second order in Na^{+} and first order in Ca^{2+}. In liver, transport via this mechanism has been fit to the equation

$$v = V_{max}\{[Na^{+}]^2/(K_{Na}^2 + [Na^{+}]^2)\} \times \{[Ca^{2+}]/(K_{Ca} + [Ca^{2+}])\} \quad (8)$$

where the best fit values of V_{max}, K_{Na}, and K_{Ca} are 2.6 ± 0.5 nmol/mg · min, 9.4 ± 0.6 mM, and 8.1 ± 1.4 nmol/mg protein, respectively (Wingrove and Gunter, 1986b). The Na^{+}-dependent mechanism is much faster in heart and brain mitochondria than in liver mitochondria; in heart the V_{max} has been reported to be 18 nmol/m 9 · min (Crompton *et al.*, 1978), while in brain the V_{max} has been found to be around 30 nmol/mg · min (Gavin, 1991).

Until recently, there has been consensus in identifying the Na^{+}-dependent Ca^{2+} efflux mechanism as a passive nonelectrogenic $Ca^{2+}/2Na^{+}$ exchanger. This conclusion was undoubtedly influenced by the transport kinetics results (Crompton *et al.*, 1977; Wingrove and Gunter, 1986b), but was particularly strongly supported by the results of Brand (1985b). In this work, a mathematically based physical chemical approach was used to show that the equilibrium Ca^{2+} distribution reached by the endogenous Na^{+}-dependent mechanism could not be distinguished from that attained in the presence of A23187 (an ionophore which electroneutrally exchanges Ca^{2+} for $2H^{+}$ ions). Since the mitochondrion possesses a very rapid Na^{+}/H^{+} exchanger (Brierley and Jung, 1988; Garlid, 1988), exchange of Ca^{2+} for 2 Na^{+} ions is thermodynamically equivalent to exchange of Ca^{2+} for 2 H^{+} ions. This led to the conclusion that the Na^{2+}-dependent mechanism was a $Ca^{2+}/2Na^{+}$ exchanger. This conclusion was further supported by the results of the reconstitution studies of Li *et al.* (1992), which were consistent with the Na^{+}-dependent mechanism being a $Ca^{2+}/2Na^{+}$ exchanger.

However, Baysal *et al.* (1991) found that the Ca^{2+} efflux via this mechanism was increased by addition of nigericin, which could be shown to have the expected effect of decreasing ΔpH and increasing $\Delta\psi$. This suggested an electrogenic component for transport via this mechanism (Baysal *et al.*, 1991). Baysal *et al.* (1994), using an approach similar to that of Gunter *et al.* (1991) (discussed above) on the Na^{+}-independent efflux mechanism, determined that, like the Na^{+}-independent mechanism, the Na^{+}-dependent mechanism has more energy available to it than can be explained if the mechanism functions as a nonelectrogenic exchanger. Their conclusion was that while the Na^{+}-dependent mechanism cannot be a simple passive $Ca^{2+}/2Na^{+}$ exchanger, it could be either a $Ca^{2+}/3Na^{+}$ exchanger (either passive or active) or an active $Ca^{2+}/2Na^{+}$ mechanism (Baysal *et al.*, 1994). See the accompanying article by Brierley *et al.* for a more complete discussion of this work.

The earlier results of Brand (1985b) were then reevaluated by Jung and coworkers (see description by Brierley *et al.* in this volume) using recently developed fluorescence techniques to evaluate intramitochondrial pH. They found that while Brand's approach was conceptually sound, there were problems with some of the assumptions and approximations used. The most troublesome parameter was intramitochondrial pH. When Jung and coworkers used their measured values for intramitochondrial pH and other parameters in Brand's equations they found that, under experimental conditions chosen to be like those of Brand, both the predicted and observed change caused by A23187 on the equilibrium reached through Ca^{2+} efflux via the Na^{+}-dependent Ca^{2+} efflux mechanism was insignificant, in contradistinction to Brand's conclusions. Furthermore, under other conditions, the results of Jung and coworkers indicated that the stoichiometry of this transporter was at least 3 Na^{+} ions per Ca^{2+} ion. While the work of Jung and coworkers did not rule out the possibility that the Na^{+}-dependent

mechanism is active, it suggests that it is not, since a passive $Ca^{2+}/3Na^{+}$ exchanger can account for their data. These recent results reopen the issue of the stoichiometry and energy supply for this mechanism and suggest that the Na^{+}-dependent Ca^{2+} efflux mechanism may function under some conditions as a $Ca^{2+}/3Na^{+}$ exchanger. It is important to try to understand why the reconsitituted system functions as a 2:1 exchanger and why the kinetics both in heart (Crompton *et al.*, 1977) and in liver (Wingrove and Gunter, 1986b) are first order in Ca^{2+} and second order in Na^{+}. Brierley *et al.* (this volume) suggest that the stoichiometry of this mechanism may vary with conditions.

SUMMARY

Mitochondria possess an elaborate Ca^{2+} transport system composed of at least three mechanisms: a uniporter and both Na^{+}-dependent and Na^{+}-independent efflux mechanisms. The uniporter, which is responsible for Ca^{2+} influx under physiological conditions, facilitates the movement of Ca^{2+} down its electrochemical gradient into the mitochondrial matrix. The uniporter is capable of sequestering Ca^{2+} from physiological pulses of Ca^{2+} within the cytosol in order to regulate metabolism (as discussed in the articles by Sheu and Jou and by Hansford in this volume).

The Na^{+}-dependent Ca^{2+} efflux mechanism is responsible for most of the mitochondrial Ca^{2+} efflux in a majority of types of vertebrate tissue. It is the greater velocity of this mechanism in heart and brain mitochondria, for example, which is responsible for the faster metabolic response to changes in work load in these tissues. There is new evidence that under some conditions, this mechanism may function as a Ca^{2+}/nNa^{+} exchanger with an n of at least 3. This new evidence must be considered carefully in view of the reported kinetics of this mechanism and evidence from the reconstituted system.

The Na^{+}-independent Ca^{2+} efflux mechanism is the most prominent mechanism of Ca^{2+} efflux from mitochondria in tissues such as liver and kidney, where very rapid metabolic response is not as necessary as it is in heart. This mechanism likely functions as an active $Ca^{2+}/2H^{+}$ exchanger.

These three mechanisms function together to control the $[Ca^{2+}]$ of the mitochondrial matrix and hence metabolic rate (as discussed in the article by Hansford in this volume) and to provide other advantages to the vertebrate cell (as discussed by Gunter *et al.*, 1994).

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