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Ba^{2+} Uptake and the Inhibition by Ba^{2+} of K^+ Flux into Rat Liver Mitochondria

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Summary. Rapid uptake of Ba^{2+} by respiring rat liver mitochondria is accompanied by a transient stimulation of respiration. Following accumulation of Ba^{2+}, e.g. at a concentration of 120 nmol per mg protein, the mitochondria exhibit reduced rates of state 3 and uncoupler-stimulated respiration. ADP-stimulated respiration is inhibited at a lower concentration of Ba^{2+} than is required to affect uncoupler-stimulated respiration, suggesting a distinct effect of Ba^{2+} on mechanisms involved in synthesis of ATP. Ba^{2+}, which has an ionic radius similar to that of K^+, inhibits unidirectional K^+ flux into respiring rat liver mitochondria. This effect on K^+ influx is observable at concentrations of Ba^{2+}, e.g. 23 to 37 nmol per mg protein, which cause no significant change in state 4 or uncoupler-stimulated respiration. The accumulated Ba^{2+} decreases the measured V_{max} of K^+ influx, while having little effect on the apparent K_m for K^+. The inhibition of K^+ influx by Ba^{2+} is seen in the presence and absence of mersalyl, an activator of K^+ influx. In contrast, under the conditions studied, Ba^{2+} has no apparent effect on the rate of unidirectional K^+ efflux. These data are consistent with the idea that K^+ may enter and leave mitochondria via separate mechanisms.

Key Words Ba^{2+} · K^+ flux · mitochondria · transport

Introduction

Ba^{2+}, which has a crystal ionic radius essentially the same as that of K^+, inhibits K^+ transport through various plasma membrane K^+ channels. These include the delayed rectifier, the inward (anomalous) rectifier, and the Ca^{2+}-dependent K^+ channels of nerve and muscle membranes (for a review *see* Latorre & Miller, 1983), as well as the apical K^+ channels of frog skin (Zieske & Van Driessche, 1983). Ba^{2+} appears to bind deep within K^+ channels of axonal membranes, perhaps at the "selectivity filter," proposed to be a negatively charged region of diameter comparable to that of the unhydrated K^+ ion (Armstrong & Taylor, 1980; Eaton & Brodwick, 1980; Latorre & Miller, 1983).

Unidirectional K^+ flux into respiring mitochondria is mediated by a mechanism which is saturable, pH-dependent, and competitively inhibited by the K^+ analog Tl^+ (Diwan & Lehrer, 1977, 1978; Jung, Chavez & Brierley, 1977). The rate of K^+ entry is increased by treatment with various thiol reactive reagents including mercurials (Gamble, 1957; Brierley, Knight & Settlemire, 1968; Diwan, Markoff & Lehrer, 1977; Jung et al., 1977), Cd^{2+} (Rasheed, Diwan & Sanadi, 1984), dibutylchloromethyltin chloride (Diwan, DeLucia & Rose, 1983) and phenylarsine oxide (Sanadi, Hughes & Joshi, 1981; Srivastava & Diwan, 1983).

It has been postulated that K^+ may enter and leave mitochondria via separate mechanisms. A K^+ uniporter is proposed to mediate electrophoretic K^+ entry while a K^+/H^+ exchanger is proposed to catalyze K^+ efflux (Mitchell & Moyle, 1969; Jung et al., 1977). The measured pH dependence of unidirectional and net K^+ efflux rates (Chavez, Jung & Brierley, 1977; Diwan & Lehrer, 1978; Diwan, 1981; Bernardi & Azzone, 1983) does not support a passive K^+/H^+ exchange mechanism. However, such a model does provide an explanation, consistent with the chemiosmotic theory (Mitchell, 1961), of observations (Diwan & Tedeschi, 1975; Diwan et al., 1979; Skulskii, Saris & Glasunov, 1983) that both influx and efflux of K^+ and other cations are respiration dependent. Treatments which cause depletion of endogenous Mg^{2+} cause an increased K^+ permeability, which has been attributed to release of regulation by Mg^{2+} of the proposed K^+/H^+ exchanger (Azzone, Bortolotto & Zanotti, 1978; Garlid, 1980; Jung et al., 1981; Nakashima, Dordick & Garlid, 1982; Bernardi & Azzone, 1983). An 82,000 dalton mitochondrial protein, identified on the basis of the observed Mg^{2+} dependence of its reactivity with [^{14}C]dicyclohexylcarbodiimide, has been proposed to have a role in K^+/H^+ exchange (Martin, Beavis & Garlid, 1984).

Oxidation of cytochrome *b* upon addition of Ba^{2+} to respiring mitochondria has been found to correlate with uptake of Ba^{2+} detected by means of

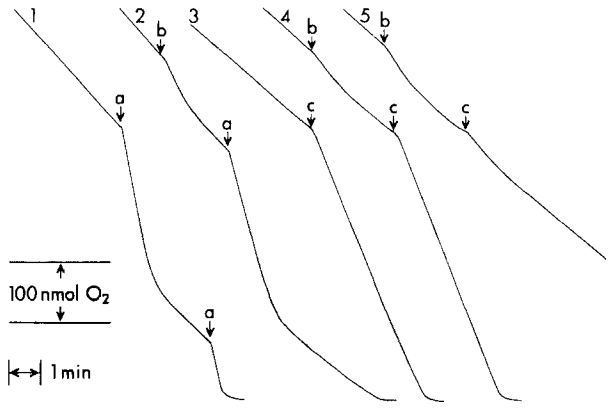

Fig. 1. Effect of Ba^{2+} on mitochondrial respiration. For samples 1 & 2 the medium included 4 mM KH_2PO_4; while for samples 3–5 4 mM KCl was substituted. The mitochondrial protein concentration was 2.48 mg per ml. Additions: *a*, 0.3 mM Tris ADP; *b*, Ba^{2+} at 150 μM in samples 2 & 4 or 300 μM in sample 5; *c*, 75 nM carbonylcyanide *m*-chlorophenylhydrazone (CCP)

the indicator dye murexide (Vainio, Mela & Chance, 1970). K^+ at 20 mM was found to block the effect of Ba^{2+} on cytochrome *b* oxidation, while having little effect on the interaction of Ca^{2+} with mitochondria (Vainio et al., 1970). It was suggested that the inhibitory effect of K^+ might be attributable to interference with surface binding of Ba^{2+} (Vainio et al., 1970; Saris & Åkerman, 1980).

Materials and Methods

Rat liver mitochondria were isolated in 0.25 M sucrose by standard procedures. Mitochondrial protein was assayed by the biuret method, using bovine serum albumin as standard (Layne, 1957). Respiration was monitored by means of a membrane-covered oxygen electrode connected to a potentiometric recorder. Mitochondria were incubated at 20°C, in a total volume of 3 ml, in medium containing (in mM): 200 sucrose, 3 succinic acid, 20 Tris, and 4 KCl or 4 KH_2PO_4, with the pH adjusted to 7.5 with HCl.

For the K^+ flux measurements, mitochondria (4 to 6 mg protein per ml) were incubated at 20°C in medium containing 200 mM sucrose, succinic acid (3 mM in experiments of Figs. 1 and 5; 7.5 mM in expts. of Fig. 2 & Table 2), 15 mM Tris, K^+ (added as KCl and as carrier with the ^{42}K; *see* figure and table legends), ^3H_2O (approx. 5 μCi/ml), ^{42}K (approx. 0.4 μCi/ml), and in some experiments [^{14}C]sucrose (approx. 0.8 μCi/ml), with the pH adjusted to 7.5 with HCl. At timed intervals mitochondrial samples were separated from incubation media by rapid centrifugation through silicone (Harris & VanDam, 1968). ^{42}K was assayed by liquid scintillation counting of the Cerenkov radiation in aqueous dilutions of mitochondrial and supernatant samples, and the counts were corrected for decay. Following decay of the ^{42}K, total K^+ levels were assayed by atomic absorption spectroscopy, and ^3H and ^{14}C were counted using a standard liquid scintillation cocktail. Fluid compartments and mitochondrial contents of total and labeled K^+ were calculated as in earlier studies (Diwan et al., 1979). Unidirectional K^+ influx rates were determined from the change in labeled K^+ content between samples taken after 0.5 and 7 min of incubation. Values of net K^+ flux were determined from the change in total K^- during the same time period. Unidirectional K^+ efflux rates represent the difference between measured influx and net flux rates.

Measurements of uptake of ^{133}Ba were carried out using similar techniques, with an equivalent incubation medium except that ^{42}K and [^{14}C]sucrose were omitted, and the medium contained 150 μM $BaCl_2$ with approx. 0.25 μCi/ml ^{133}Ba. The succinate concentration was 3.2 mM.

Radioisotopes were obtained from New England Nuclear. Silicone (SF1230) was a gift from the General Electric Co. $BaCl_2$ was obtained from Aldrich Chemical Co., and all other chemicals were from Sigma Chemical Co. or Fisher Scientific Co.

Results

Mitochondrial Respiration and Ba^{2+} Uptake

When Ba^{2+} is added to mitochondria respiring in state 4, there is a brief stimulation of respiration followed by a return to essentially the initial state 4 rate, as shown in Fig. 1 and Table 1. The respiratory burst is similar to that which accompanies Ca^{2+} uptake by mitochondria (Saris & Åkerman, 1980). Under the conditions of the experiment depicted in Fig. 1, the duration of the respiratory stimulation is about 1 min or less. The respiratory burst is observed both in the presence and absence of phosphate, although the magnitude of the stimulated rate is greater in media containing phosphate.

That Ba^{2+} is rapidly taken up by respiring mitochondria is directly confirmed, using the radioisotope ^{133}Ba, in the experiment depicted in Fig. 2. Under the conditions of the experiment in Fig. 2 (medium similar to that used for the K^+ flux measurements to be described below, including 4 mM K^+), uptake of Ba^{2+} is about 88% complete within 1 min of incubation. Steady state is reached between the 1- and 2-min samples. At the steady state, the average concentration of labeled Ba^{2+} in the medium is decreased from the initial 150 μM to 33 μM. As shown in Fig. 2, inhibition of respiration by antimycin A slows the uptake of Ba^{2+}. However, by 7 min of incubation the amount accumulated approaches that of controls. The rate of uptake of Ba^{2+} also decreases with increasing K^+ concentration in the medium. For example, in the same experiment as that of Fig. 2, the average initial Ba^{2+} uptake (0.33 to 0.40 min) was 12.4 nmol per mg protein in medium with 1 mM K^+ compared to 7.8 nmol per mg protein with 14 mM K^+. However, even in the presence of 14 mM K^+, Ba^{2+} uptake was essentially at the steady-state level within 2 min.

The data summarized in Table 1A indicate that when ADP is added following completion of the Ba^{2+}-induced respiratory burst, the state 3 respira-

Table 1. Effect of Ba^{2+} on mitochondrial respiration[a]

	mg/ml protein	μM Ba^{2+}	nmol/mg Ba^{2+}	nmol O_2/mg protein (min)		
				Initial rate	Final rate after Ba^{2+}	Rate after ADP or CCP
A	1.24	—	—	7.0		36.1
				7.6		40.6
	1.24	150	121	6.7	6.9	15.8
				6.9	7.2	16.2
	2.48	—	—	8.5		41.4
				7.8		37.7
	2.48	150	60.5	6.8	6.6	24.0
				7.9	7.4	26.6
	2.48	300	121	7.7	7.4	17.5
				7.7	7.2	16.3
B	1.24	—	—	6.3		23.2
				4.8		20.8
	1.24	150	121	6.5	6.1	15.9 → 5.0
				6.6	6.6	17.1 → 7.8
	2.48	—	—	6.7		18.6
				6.4		18.5
	2.48	150	60.5	6.6	6.1	18.4
				6.7	6.0	18.6
	2.48	300	121	6.4	6.4	8.1 → 5.8
				6.7	6.5	10.3 → 6.0

[a] The medium in A included 4 mM KH_2PO_4; and 0.3 mM Tris ADP was added to obtain the state 3 rate. The medium in B substituted 4 mM KCl; and 75 nM carbonyl cyanide *m*-chlorophenylhydrazone (CCP) was added in place of the ADP. $BaCl_2$ concentrations are given as μM and as nmol Ba^{2+} per mg mitochondrial protein. Respiration rates given are initial rates (before addition of $BaCl_2$), rates obtained after cessation of the respiratory burst induced by Ba^{2+}, and rates observed following the subsequent addition of ADP or CCP. With 121 nmol of Ba^{2+} per mg protein, the respiratory rate following addition of CCP decreased rapidly following an initial stimulation, so approximate values of initial and final rates are given. The data summarize results from the same experiment as that of Fig. 1.

tion rate is less than that of control mitochondria. If the concentration of mitochondria is varied, the extent of inhibition of state 3 respiration is found to correlate with the nanomoles of Ba^{2+} added per mg protein, rather than the μM Ba^{2+} concentration. This is not surprising since the results suggest that a major part of the Ba^{2+} added is within the mitochondria when the ADP is added.

For the samples in Table 1B, phosphate is omitted from the medium and the effect of Ba^{2+} on uncoupler-stimulated respiration is examined. The concentration of Ba^{2+} required to inhibit uncoupler-stimulated respiration is higher than that found to significantly affect ADP-stimulated respiration. At about 60 nmol Ba^{2+} per mg protein there is consistently no effect on uncoupler-stimulated respiration, while the increment of ADP-stimulated respiration is reduced, e.g. by 43% in the experiment of Table 1.

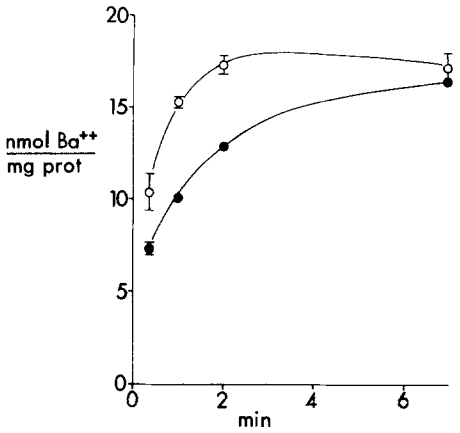

Fig. 2. Time course of ^{133}Ba uptake. The labeled Ba^{2+} taken up in units of nmol per mg protein is plotted against incubation time in minutes. The values are not corrected for contaminating external ^{133}Ba because an upper limit based on the product of the mitochondrial water space and the measured concentration of ^{133}Ba remaining in the medium was judged to be negligible for all samples (less than 0.25 nmol/mg). The medium included 4 mM KCl. Values are means of three determinations, with standard deviations shown except where less than the diameter of the symbol. Symbols: ○, control samples; ●, the medium included antimycin A at 33 ng per mg protein

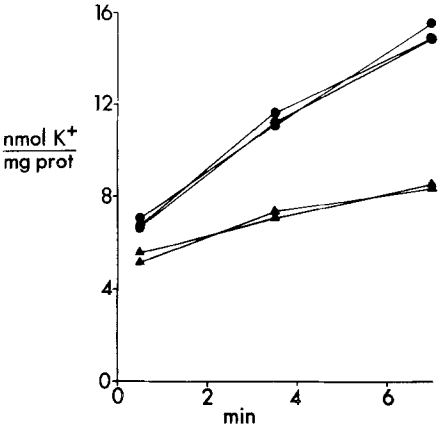

Fig. 3. Effect of Ba^{2+} on the time course of ^{42}K uptake. The mitochondrial content of labeled K^+, in units of nanomoles per mg protein, is plotted against the incubation time in minutes. The values of labeled K^+ are corrected for contaminating external K^+, estimated from the product of the measured [^{14}C]sucrose distribution space and the supernatant K^+ concentration (3.2 mM). Symbols: ●, controls; ▲, the medium included 150 μM $BaCl_2$ (37 nmol Ba^{2+} per mg protein)

K^+ Influx

The time course of ^{42}K uptake by respiring mitochondria is shown in Fig. 3. During the 7-min incu-

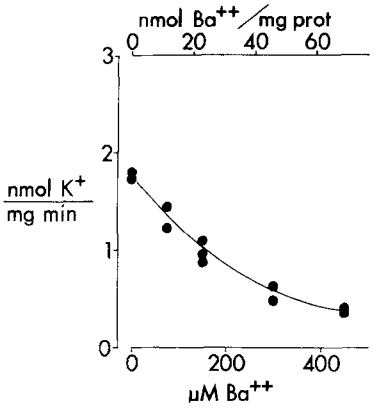

Fig. 4. Effect of varied Ba^{2+} concentration on K^+ influx. The rate of K^+ influx, in units of nmol K^+ per mg protein per min, is plotted against the concentration of Ba^{2+} included in the medium, in units of nmol per mg protein and μM. The measured K^+ concentration in the medium was 4.3 mM

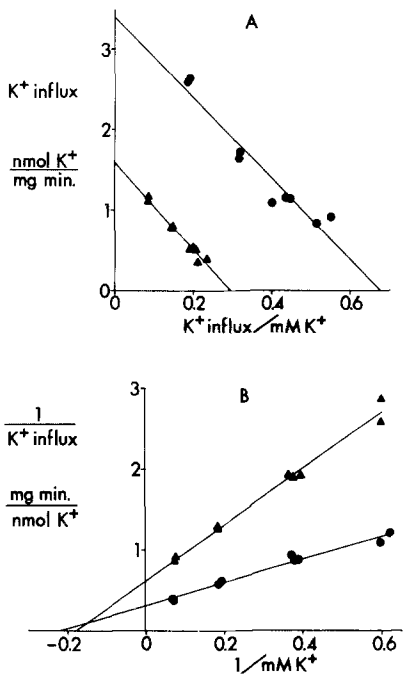

Fig. 5. Effect of Ba^{2+} on the dependence of K^+ influx on external K^+. The measured K^+ concentration of the medium varied from 1.6 to 13.8 mM. In *A* (Eadie-Hofstee plot) the K^+ influx rate, in units of nmol K^+ per mg protein per min, is plotted against the K^+ influx rate divided by the external mM K^+ concentration, in units of μl/mg protein (min). In *B* (Lineweaver-Burk plot) the reciprocal of the K^+ influx rate, in units of mg protein (min) per nmol K^+, is plotted against the reciprocal of the external K^+ concentration, in units of $(mM)^{-1}$. The lines drawn are calculated from the data by the method of least squares. Symbols: ●, control samples; ▲, the medium included 150 μM $BaCl_2$ (35 nmol Ba^{2+} per mg protein)

bations the ^{42}K uptake is approximately linear with respect to time and represents a small amount of turnover of the measured endogenous K^+ content of approximately 100 nmol per mg protein, in agreement with earlier studies (Diwan & Lehrer, 1978). The unidirectional K^+ influx rate, corresponding to the slope of the uptake curve, is reduced in the presence of 150 μM Ba^{2+} (37 nmol/mg protein). If one extrapolates the ^{42}K uptake to zero time, it is apparent that Ba^{2+} also decreases the initial rapid K^+ binding. This component of ^{42}K uptake, which is not respiration dependent, is assumed to be attributable to surface adsorption (Diwan & Lehrer, 1978). Ba^{2+} consistently causes less inhibition of K^+ adsorption than of the progressive K^+ influx. For example, for the data depicted in Fig. 3, the average K^+ influx rate is found to be inhibited 61% by Ba^{2+}, while the average extrapolated zero time K^+ binding is inhibited 17%.

The dependence on Ba^{2+} concentration of the effect of Ba^{2+} on K^+ influx is depicted in Fig. 4. The inhibition of K^+ influx increases as the Ba^{2+} concentration is increased, with 78% inhibition being obtained at the highest Ba^{2+} concentration tested (450 μM or 69 nmol/mg protein). A comparison of percent inhibition values from different experiments in which the concentration of mitochondria varied suggests that, as with effects on respiration, the extent of inhibition by Ba^{2+} varies with the nanomoles of Ba^{2+} added per mg protein, at constant μM Ba^{2+} concentration. K^+ influx is a slow process, and although respiration dependent (Diwan & Tedeschi, 1975), is not accompanied by a measurable stimulation of respiration. Results such as those depicted in Table 1 show little effect of Ba^{2+} on state 4 respiration. Nevertheless, given the observed effect on uncoupler-stimulated respiration, the possibility must be considered that at high concentrations of Ba^{2+} inhibition of K^+ influx may be due to an effect on respiration rather than to a direct effect on the K^+ transport mechanism. For the other studies of K^+ influx, effects of Ba^{2+} have been tested at 150 μM. At the concentration of mitochondria utilized for the K^+ flux measurements, which is necessarily greater than that utilized for respiration measurements, 150 μM corresponds to 23 to 37 nmol Ba^{2+} per mg protein. In this concentration range, the data indicate that Ba^{2+} should have little effect on respiration, except during the uptake of Ba^{2+}, which would be complete early in the 0.5- to 7-min time course of the ^{42}K influx measurements.

The dependence of K^+ influx on the K^+ concentration in the medium is examined in Fig. 5. In agreement with earlier studies, Lineweaver-Burk plots of the reciprocal of the K^+ influx rate *vs.* the reciprocal of the external K^+ concentration are linear (Diwan & Lehrer, 1978; Diwan, 1981), although

Table 2. Apparent kinetic constants[a]

	mM K^+ K_m		nmol K^+/mg prot (min) V_{max}	
	L-B	E-H	L-B	E-H
Control	3.9 ± 0.8	3.9 ± 1.1	2.8 ± 0.3	2.9 ± 0.5
+ Ba^{2+}	4.4 ± 1.1	4.5 ± 1.0	1.5 ± 0.1	1.5 ± 0.1

[a] Values are means of kinetic constants calculated from least-squares fits of data from the experiment of Fig. 5 and two other similar experiments, ± standard deviations. L-B refers to the Lineweaver-Burk and E-H to the Eadie-Hofstee transformations of the Michaelis Menton equation.

Table 3. Effect of Ba^{2+} and mersalyl on K^+ flux[a]

Additions	nmol K^+/mg protein (min)	
	K^+ influx	K^+ efflux
none	1.67 ± 0.06	1.3 ± 0.4
Ba^{2+}	0.84 ± 0.16	1.4 ± 0.5
mersalyl	2.78 ± 0.05	1.5 ± 0.3
Ba^{2+} + mersalyl	1.62 ± 0.10	1.6 ± 0.4

[a] Ba^{2+} and mersalyl when present were each included in the medium at 150 μM or 27.5 nmol per mg protein. Values are means of three determinations ± standard deviations.

a slight nonlinearity of the Eadie-Hofstee plots is sometimes observed. Apparent kinetic constants calculated from three similar experiments are summarized in Table 2. The data consistently show an essentially noncompetitive pattern of inhibition. The calculated V_{max} decreases in the presence of Ba^{2+}, while the apparent K_m for K^+ changes very little.

Table 3 examines the effect of the Ba^{2+} on K^+ influx activated by mersalyl. An enhanced rate of K^+ influx is observed in the presence of the mercurial, consistent with earlier studies (Diwan et al., 1977; Jung et al., 1977). The activated K^+ influx remains sensitive to Ba^{2+}. In the experiment of Table 3, 42% inhibition by Ba^{2+} of K^+ influx in the presence of mersalyl is observed, compared to 50% inhibition of control influx rates. In another comparable experiment, 42% inhibition by Ba^{2+} was observed in the presence of mersalyl compared to 44% inhibition in the absence of mersalyl.

Table 3 also lists unidirectional K^+ efflux rates. The results of this and other experiments show that Ba^{2+} causes no inhibition of K^+ efflux.

Discussion and Conclusions

The results have directly confirmed an earlier report of respiration-dependent uptake of Ba^{2+} by mitochondria (Vainio et al., 1970). An observed slowing of the Ba^{2+} uptake rate in the presence of K^+ is consistent with the finding that K^+ blocks the stimulation by Ba^{2+} of cytochrome *b* oxidation (Vainio et al., 1970). The current studies show that Ba^{2+}, at relatively high concentrations, inhibits state 3 respiration and uncoupler-stimulated respiration. The finding that ADP stimulation of respiration is inhibited at a concentration of Ba^{2+} which fails to affect uncoupler-stimulated respiration suggests an interaction of Ba^{2+} with the ATP synthase complex, or with the adenine nucleotide translocase, which is distinct from its interaction with the respiratory chain.

Ba^{2+} inhibits K^+ flux into respiring mitochondria. This effect is observable at Ba^{2+} concentrations which do not significantly impair mitochondrial respiration under similar conditions. The finding that K^+ adsorption is less affected by Ba^{2+} than is K^+ influx suggests that the effect of Ba^{2+} on K^+ influx is probably not attributable to interference with surface binding.

The kinetic studies suggest an essentially noncompetitive pattern of inhibition of K^+ influx by Ba^{2+}. Purely competitive inhibition might have been expected considering the similarity of size of the Ba^{2+} and K^+ ions and the evidence for competitive interactions in other transport systems (Latorre & Miller, 1983). However, it should be noted that the ^{133}Ba uptake studies, respiratory measurements, and the dependence on protein concentration of Ba^{2+} effects, indicate that the added Ba^{2+} is largely taken up by the mitochondria early in the time period of the ^{42}K uptake measurements. In the absence of an ability to measure isotope fluxes on a much faster time scale, the ^{133}Ba uptake measurements do not permit distinguishing instantaneous surface binding from respiration-linked Ba^{2+} influx. Nevertheless the similarity of the respiratory cycles observed with Ba^{2+} and Ca^{2+} suggests that a significant portion of the Ba^{2+} taken up does enter the matrix compartment. Thus the ^{42}K flux measurements are examining effects of a largely internalized and concentrated inhibitor on reaction of external K^+ with the K^+ transport mechanism. Furthermore, although in some sense a substrate analog, the divalent Ba^{2+} may not be a good substrate capable of reversibly traversing the mitochondrial K^+ channel or carrier site. In fact the reported sensitivity of Ba^{2+} uptake to Pr^{3+}, an inhibitor of the Ca^{2+} transport mechanism (Vainio et al., 1970), suggests that Ba^{2+} may enter the mitochondria via the Ca^{2+} transporter. The mitochondrial K^+ transport mech-

anism differs from the Ca^{2+} transporter in kinetic characteristics and inhibitor sensitivity (Diwan & Lehrer, 1978; Saris & Åkerman, 1980).

The mechanism of activation of K^+ influx by mersalyl remains unclear. It was earlier proposed that mercurials may enhance K^+ permeability by causing discharge of endogenous Mg^{2+} (Bogucka & Wojtczak, 1979). However, this hypothesis has not been supported by measurements of mitochondrial Mg^{2+} (Diwan, Aronson & Gonsalves, 1980) or by the observed sensitivity to mersalyl of K^+ flux into divalent cation-depleted mitochondria (Jung et al., 1981). It has been shown that mersalyl increases the V_{max} of respiration-dependent K^+ influx without altering the apparent K_m for K^+ (Diwan et al., 1977). It has been suggested that mersalyl may open up an alternative pathway for electrophoretic K^+ flux (Jung et al., 1981). The present data indicate that the K^+ translocation mechanism activated by mersalyl exhibits a sensitivity to Ba^{2+} similar to that of the native K^+ influx mechanism. In contrast, the mechanism mediating K^+ efflux appears to be insensitive to Ba^{2+}. This finding is consistent with the proposal (Mitchell & Moyle, 1969; Jung et al., 1977) that K^+ may enter and leave mitochondria via separate mechanisms.

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