

On the Relative Roles of Ca^{2+} and Mg^{2+} in Regulating the Endogenous K^+/H^+ Exchanger of Rat Liver Mitochondria*

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1. A23187 and ionomycin cause the release of Ca^{2+} , Mg^{2+} , and K^+ from the mitochondrial matrix. The electroneutral K^+ efflux does not reflect direct transport by these ionophores but, rather, results from release of the endogenous K^+/H^+ exchanger from inhibition by divalent cations.

2. A23187 and ionomycin differ in their affinities for Ca^{2+} and Mg^{2+} , having relative affinity ratios ($\text{Ca}^{2+}/\text{Mg}^{2+}$) in respiring mitochondria of 3 and 35, respectively.

3. This difference in $\text{Ca}^{2+}/\text{Mg}^{2+}$ affinities was exploited in dose-response studies designed to determine which cation exerts primary control over K^+/H^+ exchange activity. The results of such studies demonstrate that removal of Ca^{2+} has no effect on K^+ efflux, and that K^+ efflux follows Mg^{2+} efflux whether induced by ionomycin or A23187.

4. K^+ steady states, in which K^+ uniport equals K^+/H^+ antiport, were induced by adding low levels of valinomycin to respiring mitochondria. The addition of Ca^{2+} perturbed the steady state in the direction of increased K^+/H^+ exchange.

5. Addition of Ca^{2+} to respiring mitochondria resulted in electroneutral K^+ efflux which was strongly affected by the anion composition of the medium. We propose that Ca^{2+} turns on the K^+/H^+ exchanger indirectly, through a decrease in matrix $[\text{Mg}^{2+}]$ secondary to swelling and uptake of chelating anions. This phenomenon may be of physiological importance as a temporary protection against excessive swelling due to Ca^{2+} uptake.

6. These studies support the contention that Mg^{2+} , not Ca^{2+} , is the inhibitory cation responsible for the physiological regulation of mitochondrial K^+/H^+ exchange.

identification of Mg^{2+} , rather than Ca^{2+} , as the modulator of K^+/H^+ exchange was based upon physiological considerations; since the rate of exchange is determined by the activity of matrix M^{2+} , the feedback mechanism will work most efficiently if the total amount of M^{2+} remains relatively constant (5). It is well established that mitochondria possess a mechanism for rapid Ca^{2+} uptake (8-10), and it has been proposed that mitochondria function as physiological modulators of intracellular Ca^{2+} activity (11-14). In comparison, Mg^{2+} transport in rat liver mitochondria is extremely limited (15-17), suggesting that Mg^{2+} would be a more efficient regulator of K^+/H^+ exchange than Ca^{2+} . Contrary to these expectations, Ca^{2+} is found to block K^+ efflux more effectively than Mg^{2+} in mitochondria depleted of divalent cations by pretreatment with A23187 (7). Aside from this finding in a nonphysiological state, there is no experimental evidence in the literature regarding the relative roles of Mg^{2+} versus Ca^{2+} in the regulation of the K^+/H^+ exchanger. The present report addresses this question by examining the effects of altered matrix $[\text{Ca}^{2+}]$ on K^+/H^+ exchange activity.

In the first part of this study, we exploited the difference in $\text{Ca}^{2+}/\text{Mg}^{2+}$ affinity ratios of A23187 and ionomycin to determine the effect of selective loss of Ca^{2+} on K^+/H^+ exchange. Conclusions from these experiments depend on the assumption that the observed K^+ efflux is mediated by the endogenous exchanger, and not by direct transport on the divalent cation ionophores, as has been previously reported (18, 19). We have demonstrated that A23187 does not transport K^+ directly (6, 7), and the present study supports a similar conclusion for ionomycin. A comparison of the dose-response curves for K^+ loss induced by ionomycin and A23187 strongly suggests that K^+ efflux depends on depletion of Mg^{2+} , rather than Ca^{2+} .

In the second part of this study, we examined the effects of Ca^{2+} uptake on K^+/H^+ exchange in respiring mitochondria. Depending on the anion composition of the medium, Ca^{2+} addition induced a pronounced, electroneutral K^+ efflux. We infer from these studies that Mg^{2+} , not Ca^{2+} , is responsible for inhibition of mitochondrial K^+/H^+ exchange under *in vivo* conditions.

EXPERIMENTAL PROCEDURES

General Methodology—Rat liver mitochondria were isolated using the high yield, differential centrifugation procedure of Pedersen *et al.* (20). Stock suspensions containing 50 mg of protein/ml were stored in 0.25 M sucrose at 0 °C. All experiments were carried out at 25 °C.

Ion Electrode Studies—Oxygenated suspensions of mitochondria were monitored with a Corning model 476220 monovalent cation electrode. Potential readings were obtained at 6-s intervals and converted, through standards analyses, to $[\text{K}^+]_o$ (external K^+ , mM) and \bar{K}_m^+ (matrix K^+ , nmol/mg) as described (6).

Dose-Response Studies—1 ml of stock suspension was treated with rotenone (2 nmol/mg) and allowed to warm at room temperature for 1 min. This aliquot was then mixed with 3 ml of oxygenated incubation

The mitochondrial K^+/H^+ exchanger has been assigned a primary role in regulation of matrix volume (1-5). It has been demonstrated that the rate of K^+/H^+ exchange is correlated with divalent cation activities in the matrix (5-7), and this has led us to propose an "Mg²⁺ carrier brake" model for mitochondrial volume homeostasis (see Fig. 1 and Ref. 5). The

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1 The abbreviations used are: M^{2+} , divalent cation; TEA⁺, tetraethylammonium ion.

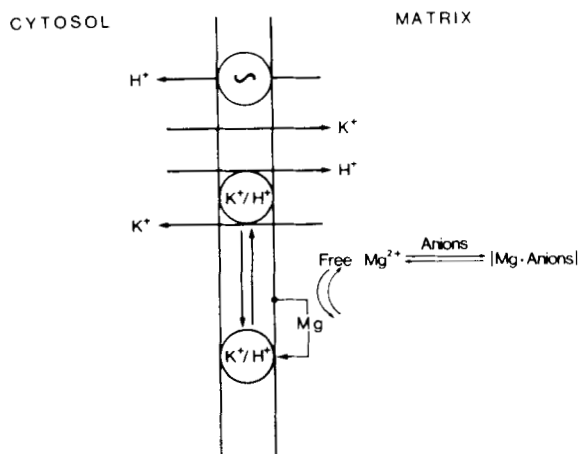


FIG. 1. The Mg^{2+} carrier brake model for regulation of the mitochondrial K^+/H^+ exchanger. See text for discussion.

solution containing sucrose (120 mM), $CaCl_2$ (10 nmol/mg), tetraethylammonium (TEA^+) salts of P_i (0.4 mM), succinate (10 mM), malate (0.1 mM), acetate (49 mM), and *N*-(tris(hydroxymethyl)methyl-2-amino)ethanesulfonic acid (20 mM, pH 7.2). Incubations were carried out in a shaking water bath at 25 °C. After 1 min, ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid and EDTA were added to a final concentration of 40 and 80 nmol/mg of protein, respectively. 1 min later, 5 to 15 μ l of dimethylsulfoxide containing the desired dose of ionophore were added to the mix. After 1 min of exposure to ionophore, the mitochondria were centrifuged at 20,000 $\times g$ for 3 min. Supernatants and pellets were separately extracted as in Ref. 5 and assayed for Mg^{2+} , Ca^{2+} , and K^+ by atomic absorption spectroscopy (Perkin-Elmer model 403).

Control mitochondria were found to contain 12, 29, and 110 nmol/mg of Ca^{2+} , Mg^{2+} , and K^+ , respectively. Losses due to ionophore treatment were scaled according to maximal losses observed. The latter were very similar for A23187 and ionomycin, respectively: 92% and 89% of Mg^{2+} , 98% and 97% of Ca^{2+} , and 77% and 76% of K^+ . This agreement shows that no artifacts were introduced through the scaling method. The incomplete loss of K^+ is due to the time interval (1 min) selected. 100% loss of K^+ occurs within 75 s at high ionophore doses under these conditions.

Solutions and Chemicals— TEA^+ and trimethylamine salts were prepared by neutralization of the respective acids by the free base. Nigericin, valinomycin (Calbiochem-Behring), A23187 (Calbiochem-Behring), and ionomycin solutions were gravimetrically prepared in dimethylsulfoxide. Ionomycin and nigericin were the generous gifts of Dr. Edward Meyers (Squibb Institute for Medical Research, Princeton, NJ) and Dr. W. E. Scott (Hoffmann-LaRoche, Nutley, NJ), respectively.

RESULTS

Ionomycin-induced K^+ Loss—The data in Fig. 2 illustrate the basic phenomena of spontaneous and ionophore-induced electroneutral K^+ efflux. Mitochondria respiring in TEA^+ salts lose K^+ slowly, until TEA^+ uptake and swelling result in spontaneous release of the K^+/H^+ exchange mechanism (4). When ionomycin is added, a rapid K^+ efflux ensues within 30 s (see Fig. 2). K^+ loss is reversed by valinomycin in both cases, indicating that the K^+ efflux is electroneutral. K^+ efflux induced by ionomycin differs from the spontaneous K^+ efflux in the following significant respects. The rate is more rapid. The lag time is shorter and depends on the dose of ionophore used. The efflux is not anion-dependent; in particular, it is not inhibited by acetate. These characteristics of ionomycin-induced K^+ efflux are identical with those previously described for A23187-treated preparations (6). The simplest explanation for these observations is that mitochondria possess an endogenous K^+/H^+ exchanger whose rate is regulated by divalent cations (5).

Ionomycin Does Not Transport K^+ —The use of divalent cation ionophores to unmask the endogenous K^+/H^+ ex-

changer provides a powerful experimental tool for the study of this process. However, it must be shown that the ionophore itself does not transport K^+ . Because of a report to the contrary (19), it was necessary to re-examine the mechanism by which ionomycin induces K^+ efflux. The results shown in Fig. 3 represent K^+ efflux rates as a function of dose of A23187 and ionomycin. The data show that both ionophores induce a plateau in the rate of K^+ efflux of similar magnitude. 50% of maximal rate was achieved at about 0.15 nmol/mg of A23187 and 1.7 nmol/mg of ionomycin. The maximal rates induced by the two ionophores are not identical; however, since A23187 was added as the free acid and ionomycin as a calcium salt, we consider the difference in plateaus between ionomycin and A23187 to be within the range to be expected. Under identical conditions, nigericin induced rates of K^+ efflux which were several times that observed with A23187 or ionomycin (not shown). This finding establishes that the K^+ efflux in Fig. 3 is not limited by gradients for K^+/H^+ exchange. The fact that

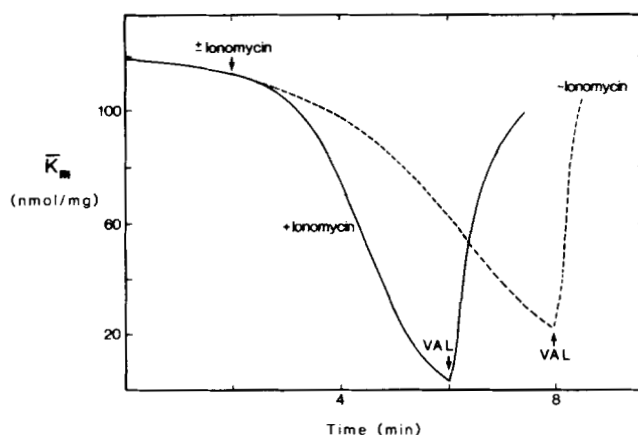


FIG. 2. Ionomycin-induced K^+ efflux from respiring mitochondria. Following pretreatment with rotenone (2 nmol/mg), mitochondria (2.9 mg/ml) were suspended in a medium containing 0.28 mM KCl, the TEA^+ salts of succinate (10 mM), phosphate (1 mM), malate (1 mM), *N*-(tris(hydroxymethyl)methyl-2-amino)ethanesulfonic acid (pH 7.2, 20 mM), EDTA (0.48 mM), ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (0.38 mM), and chloride (17.8 mM), made isotonic (0.272 osmolal) with sucrose. Valinomycin (0.5 nmol/mg) was added as indicated (VAL). Data are plotted as matrix K^+ (nanomoles/mg) versus time.

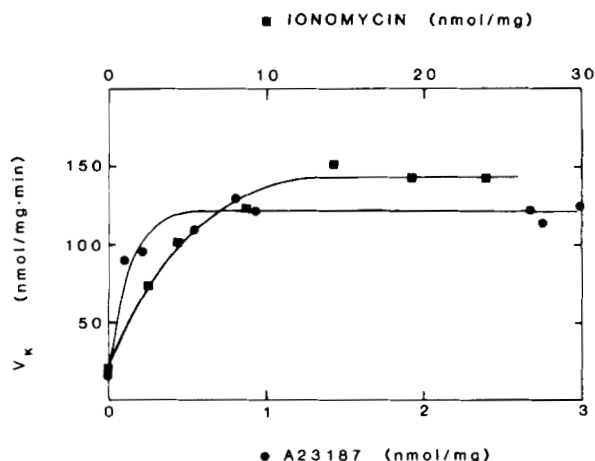


FIG. 3. The rate of K^+ efflux as a function of dose of A23187 and ionomycin. Conditions were as described in the legend to Fig. 2. The various dosages of A23187 and ionomycin were added to the suspension 1.5 min and 3 min, respectively, after the addition of mitochondria. The data plotted represent the maximum efflux rate, V_k , observed with the ionophore dosage.

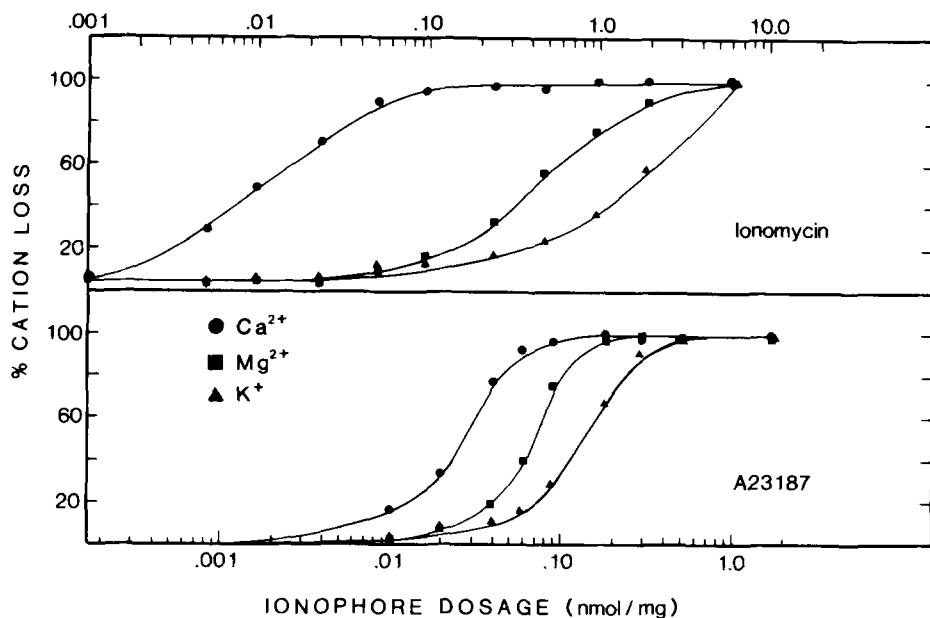


FIG. 4. Dose-response curves for Mg^{2+} , Ca^{2+} , and K^+ loss induced by A23187 and ionomycin. For conditions see under "Experimental Procedures." The percentage of loss of mitochondrial cation after 1 min of exposure to ionophore is plotted as a function of ionophore dosage.

TABLE I

Apparent cation affinity ratios for A23187 and ionomycin in intact mitochondria

Data were obtained by comparing the doses of ionophore required to induce 50% loss of mitochondrial cation after 1 min of exposure to drug. Conditions were the same as described in the legend to Fig. 4. The results reported are based upon two experiments with ionomycin and four experiments with A23187.

Ionophore	Ca^{2+}/Mg^{2+}	Ca^{2+}/K^+	Mg^{2+}/K^+
A23187	3	5	2
Ionomycin	35	135	3

increased doses of ionomycin fail to increase the rate of K^+ efflux once the plateau has been reached (Fig. 3) argues strongly against a direct K^+ -transporting ability for either ionophore.

Steady state perturbation studies were also carried out, in which steady state K^+ levels were induced by low levels of A23187 and valinomycin in the presence of EDTA (see Ref. 6). It was found that ionomycin did not perturb this steady state (not shown), indicating that it has no K^+ -transporting ability even in the absence of divalent cations. We conclude that ionomycin induces K^+ loss by releasing the endogenous K^+/H^+ exchanger from divalent cation inhibition in a manner entirely analogous to that previously shown for A23187 (6, 7).

Dose-Response Studies on Efflux of K^+ , Mg^{2+} , and Ca^{2+} Induced by A23187 and Ionomycin—A representative set of dose-response curves for K^+ , Mg^{2+} , and Ca^{2+} efflux following 1-min exposures to A23187 and ionomycin is plotted in Fig. 4. Both ionophores remove Ca^{2+} first with a similar potency, 50% Ca^{2+} loss occurring at 0.03 nmol/mg of A23187 and 0.01 nmol/mg of ionomycin. As previously reported (19), ionomycin exhibits greater Ca^{2+}/Mg^{2+} selectivity than A23187. A comparison of the doses required to give 50% cation losses permits estimates of relative affinities for these two ionophores to be made (see Table I). These "affinity" ratios must be regarded as rough approximations to the true ratios of Ca^{2+}/Mg^{2+} , due to the complexity of the experimental system. Nevertheless, they are useful in that they represent relative potencies of these two agents in respiring mitochondria. Furthermore, the Ca^{2+}/Mg^{2+} ratio obtained with A23187 in this study is in remarkably good agreement with organic phase extraction studies of Pfeiffer and Lardy (18) which give estimates of Ca^{2+}/Mg^{2+} affinity ratio close to 3. The present study

provides an estimate of 35 for the apparent Ca^{2+}/Mg^{2+} affinity ratio for ionomycin in intact mitochondria.

The experiments of Fig. 4 were carried out in 49 mM acetate, a medium in which K^+/H^+ exchange is normally inhibited (5). Both A23187 and ionomycin release this inhibition, and a rapid K^+ efflux occurs which is reversed by valinomycin (not shown). Since neither ionomycin nor A23187 directly transport K^+ , and since K^+ efflux occurs in a direction opposite to electrophoretic K^+ flux, we conclude that this process represents electroneutral K^+/H^+ antiport on the endogenous exchanger. If Ca^{2+} played a role in blocking K^+/H^+ exchange, one would expect the dose-response curve for ionomycin to reflect this inhibition, since all Ca^{2+} is removed before Mg^{2+} levels are affected (Fig. 4). This is not the case. K^+ efflux can be seen to follow Mg^{2+} efflux following treatment with either drug, and there is no relationship between K^+ efflux and Ca^{2+} removal. This conclusion is also reflected in the data shown in Table I, in which no relationship exists between the Ca^{2+}/K^+ ratios for A23187 and ionomycin, while the Mg^{2+}/K^+ ratios are similar for the two ionophores. This study indicates that loss of matrix Ca^{2+} does not induce K^+/H^+ exchange, and that Mg^{2+} , not Ca^{2+} , is responsible for the inhibition of the exchanger which obtained prior to ionophore treatment.

Steady State Perturbation Studies—*In vivo*, the free energy gradients for electrophoretic K^+ uniport and electroneutral K^+/H^+ antiport are in opposite directions, driving electrophoretic K^+ influx (J_{K^+}) and electroneutral K^+ efflux (J_{K^0}), respectively. In the steady state, matrix K^+ is constant, and $J_{K^+} + J_{K^0} = 0$. *In vitro*, it is necessary to employ low values of $[K^+]_0$ to obtain good sensitivity with the ion electrode technique. Fortunately, the electrochemical gradient for K^+ remains inward-directed down to $[K^+]_0$ values of about 0.1 mM. This means that the *in vivo* steady state can be approximated at low $[K^+]_0$ levels *in vitro*. The reduced gradient for K^+ uptake is compensated by adding low doses of valinomycin to increase the electrophoretic component of the K^+ cycle.

Such a steady state is shown in Fig. 5A (dashed line), where matrix K^+ remained relatively stable for up to 10 min. Further K^+ uptake results when small increments of valinomycin are added (not shown), until a saturating dose (about 0.5 nmol/mg) is reached (Fig. 5A). At this point, electrophoretic K^+ flux predominates ($J_{K^+} \gg J_{K^0}$), and K^+ approaches electrochemical equilibrium across the membrane. These effects of valinomycin show that the initial plateau in Fig. 5A is a true steady

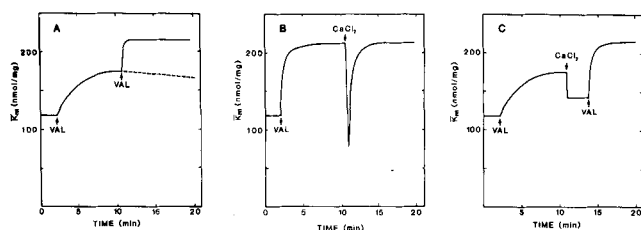


FIG. 5. Effects of Ca^{2+} on the steady state level of matrix K^+ . A, rotenone-treated mitochondria (2.8 mg/ml) were suspended in a medium containing K^+ (0.28 mM), the trimethylamine salts of succinate (5.75 mM), phosphate (1 mM), citrate (5 mM), and *N*-(tris(hydroxymethyl)methyl-2-amino)ethanesulfonic acid (pH 7.2, 17 mM), made isotonic (0.272 osmolal) with sucrose. The steady state induced by a low dose of valinomycin (3.0 pmol/mg) was maintained for 10 min (dashed line). Addition of a high dose of valinomycin (0.5 nmol/mg) during the steady state resulted in K^+ uptake (solid line). Increasing the dose of valinomycin (VAL) beyond 0.5 nmol/mg had no effect on mitochondrial K^+ levels. B, conditions were the same as in A. Valinomycin (0.5 nmol/mg) and $CaCl_2$ (160 nmol/mg) were added as indicated. C, conditions were the same as in A. After a steady state was induced with a low dose of valinomycin (3.0 pmol/mg), $CaCl_2$ (160 nmol/mg) and a high dose of valinomycin (0.5 nmol/mg) were added as indicated. During the course of these runs external $[K^+]$ varied from 0.35 mM after mitochondrial addition to 0.10 mM after the high dose of valinomycin. The Ca^{2+} effects shown in B and C reflect mitochondrial K^+ transport. The K^+ electrode does not respond to Ca^{2+} added in the absence of mitochondria or in the presence of mitochondria solubilized with Triton X-100.

state and that there is an underlying electroneutral K^+ efflux. The steady state can be perturbed in the opposite direction by adding either nigericin, a K^+/H^+ ionophore, or A23187, which increases the rate of the endogenous K^+/H^+ exchanger (6).

The effect of Ca^{2+} (160 nmol/mg) on the equilibrium distribution of K^+ is shown in Fig. 5B. Ca^{2+} caused a transient ejection of K^+ , followed by reuptake to near the original level. These findings are consistent with accepted views of Ca^{2+} transport in energized mitochondria (8). The rapid electrophoretic uptake of Ca^{2+} transiently collapses the membrane potential, leading to ejection of K^+ in the presence of a high dose of valinomycin. When Ca^{2+} accumulation is complete, the potential returns toward its previous value, and K^+ is reaccumulated.

The effect of Ca^{2+} on the steady state distribution of K^+ is shown in Fig. 5C. It can be seen that Ca^{2+} caused a shift to a lower K^+ content, and the subsequent addition of a saturating dose of valinomycin resulted in K^+ uptake (Fig. 5C). Ca^{2+} uptake by these preparations results in uptake of anions and water. The shift of steady state K^+ is in a direction which will tend to compensate for these changes, and the steady state perturbation by Ca^{2+} , therefore, represents an appropriate homeostatic response. This result suggests that Ca^{2+} stimulates, rather than inhibits, the K^+/H^+ exchanger; however, this protocol, in which both uniport and antiport are operating at high rates, does not permit a firm assignment of the Ca^{2+} effect to a single pathway.

Stimulation of K^+/H^+ Exchange by Ca^{2+} —A direct test of the effects of Ca^{2+} on K^+ transport was carried out in the absence of ionophores, where the uniport pathway is very low. Ca^{2+} (100 nmol/mg) was added to mitochondria respiring in trimethylamine salts, and K^+ movements were followed with the ion electrode. Anion composition was varied, since Ca^{2+} loading is anion-dependent (22). A representative sample of a large number of studies on Ca^{2+} -induced K^+ loss is presented in Fig. 6. This phenomenon is highly anion-dependent. Acetate inhibited Ca^{2+} -induced K^+ efflux (curve a), despite the finding by Rasmussen *et al.* (21) that acetate supports accumulation of Ca^{2+} with swelling. K^+ efflux was also reduced

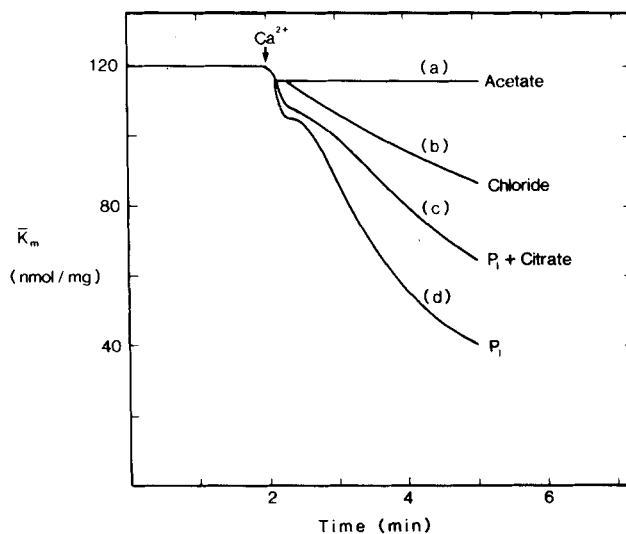


FIG. 6. Mitochondria (2.9 mg/ml) were incubated in a buffered, isotonic medium containing succinate, as described in the legend to Fig. 5. Other anions present were as follows: 8.25 mM acetate (curve a), 8.25 mM chloride (curve b), 1 mM phosphate and 3 mM citrate (curve c), and 5.5 mM phosphate (curve d). $CaCl_2$ (100 nmol/mg) was added to the suspension at 2 min. The addition of valinomycin at 5 min caused K^+ uptake in all cases (not shown).

when phosphate was omitted from the medium (curve b), a condition in which limited Ca^{2+} loading and swelling is expected (22). In contrast, Ca^{2+} -induced K^+ efflux was stimulated both by high phosphate (curve d) and by citrate in the presence of low phosphate (curve c). An important feature of these studies, omitted from Fig. 6 for clarity, is that valinomycin caused reuptake of K^+ in all cases, identifying the efflux as electroneutral. Thus, the suggestion made in the previous section is confirmed. Depending on the anion composition, Ca^{2+} stimulates, rather than inhibits, electroneutral K^+ efflux.

Additional anion-dependent phenomena have also been observed and tentatively identified, and these will be described briefly. 1) In agreement with a report by Siliprandi *et al.* (23), we have found that mitochondria lose about 20% of endogenous Mg^{2+} during respiration in succinate. This loss is accelerated by P_i and reduced by citrate. Most of the Mg^{2+} loss occurs during the first 2 min of incubation, before Ca^{2+} was added in the present studies. 2) In the presence of P_i , Ca^{2+} addition results in an immediate, rapid burst of K^+ ejection, which can be seen in the records of Fig. 6. This initial K^+ ejection is not electroneutral; rather, it is a result of the transient membrane depolarization which occurs during Ca^{2+} uptake, as evidenced by the K^+ efflux spike observed in the presence of valinomycin (see Fig. 5B). After the transient phase of Ca^{2+} uptake is completed, the driving force for K^+ uniport becomes inward again, confirmed by the finding that valinomycin causes K^+ uptake when added during this latter phase (Fig. 6).

DISCUSSION

Previous studies have indicated that the mitochondrial K^+/H^+ exchanger is reversibly inhibited by matrix divalent cations (5–7, 24–26) leading to a feedback mechanism for volume homeostasis (3–6). Physiological arguments favor Mg^{2+} over Ca^{2+} for this role (5), but there is little experimental evidence for this conclusion. Indeed, Ca^{2+} was found to be more effective than Mg^{2+} in the study by Shi *et al.* (7). The present study addresses the question whether Ca^{2+} or Mg^{2+} or both are effective inhibitors of the K^+/H^+ exchanger in the physiologically important range of activities of this transporter.

All other conditions being equal, K^+/H^+ exchange activity

should be proportional to the fraction of free exchanger, as approximated by Equation 1.

$$\text{Fraction of free exchanger} = (1 + [M^{2+}]/k)^{-1} \quad (1)$$

where $[M^{2+}]$ is the activity of divalent cation, and k is its affinity for the exchanger. Fig. 7, a hypothetical plot of K^+/H^+ exchange activity as a function of $[M^{2+}]$, is a useful guide for identifying the region of the titration curve over which variations in $[M^{2+}]$ take place. 1) The *right* region of the curve represents the state of freshly isolated mitochondria which retain K^+ despite a massive gradient favoring K^+/H^+ exchange. Hypotonic swelling reduces $[M^{2+}]$ by dilution, resulting in swelling-induced K^+ loss (5). This region of the curve must also reflect the normal steady state *in vivo*, otherwise futile cycling of K^+ would result in heat production instead of energy conservation. 2) The *central* region of the curve, where carrier activity varies most with changes in $[M^{2+}]$, may also be of physiological significance, describing a range over which the K^+/H^+ exchanger can respond rapidly to massive swelling. 3) The *left* end of the curve represents a nonphysiological region associated with total divalent cation depletion. In this region, the rate of K^+/H^+ exchange can be expected to respond to any inhibitory cation.

Matrix $[M^{2+}]$ can be varied with the aid of A23187 and ionomycin, which also induce rapid, electroneutral K^+ efflux (Fig. 2). We conclude that these ionophores do not transport K^+ directly (Fig. 3 and Refs. 6 and 7), in contrast to previous reports in the literature (18, 19). Consequently, the K^+ efflux must reflect an endogenous exchange mechanism which is under inhibitory control by divalent cations, as shown in Fig. 7. The difference in Ca^{2+}/Mg^{2+} affinity ratios of ionomycin and A23187 (19) suggested that a distinction between the effects of Ca^{2+} and Mg^{2+} might be revealed by comparing the dose-response curves for the two ionophores. As expected, K^+ loss occurred subsequent to depletion of Mg^{2+} and Ca^{2+} (see Fig. 4), and ionomycin was found to have a selectivity for Ca^{2+} much greater than that of A23187 (see Table I). It is apparent that the dose-response curve for K^+ closely followed the Mg^{2+} curve but not the Ca^{2+} curve. Total mitochondrial Ca^{2+} could be depleted to less than 1 nmol/mg of protein with little loss of K^+ , while depletion of Mg^{2+} to 10 nmol/mg resulted in rapid K^+ efflux (Fig. 4). Since the K^+/H^+ exchanger was maximally inhibited prior to ionophore addition, we infer that Mg^{2+} is the predominant regulator in this physiologically important region of low futile cycling of K^+ .

We next looked at the effects of Ca^{2+} on steady state cycling of K^+ . To achieve a steady state in low $[K^+]_0$ which is comparable to that which obtains *in vivo*, it is necessary to

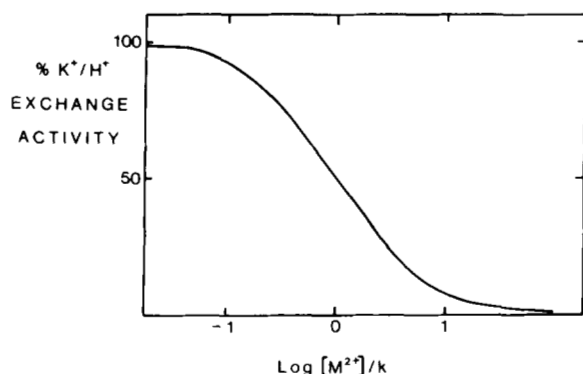


FIG. 7. Hypothetical plot of percentage of K^+/H^+ exchanger activity as a function of matrix divalent cation activity. For a description of the figure see under "Discussion."

compensate the reduced K^+ electrochemical gradient by increasing the electrophoretic permeability. This was accomplished with low doses of valinomycin (Fig. 5A). The effect of ionophore concentration confirms the existence of a steady state balance between K^+ uniport and antiport (Fig. 5A). The equilibrium distribution of K^+ is little affected by Ca^{2+} (Fig. 5B), while the steady state is shifted by Ca^{2+} in the direction of K^+ efflux and contraction (Fig. 5C). The latter result is opposite to what would be expected if Ca^{2+} inhibited the K^+/H^+ exchanger under these conditions, and suggests that mitochondria are equipped to compensate for Ca^{2+} -induced swelling.

The results in Fig. 6 demonstrate directly that Ca^{2+} can induce electroneutral K^+ efflux, depending on the anion composition of the medium. Ca^{2+} is ineffective in acetate, slightly effective in Cl^- , and very effective in P_i and citrate. It is noteworthy that these anion effects parallel those observed in previous studies on swelling-induced K^+ efflux (3, 5) and on tetraethylammonium-induced K^+ efflux (4). These results are consistent with the titration curve in Fig. 7. In acetate, the matrix is acidified and purged of endogenous anions, so that $[M^{2+}]$ is very high, and the carrier is inhibited. The lack of effect of Ca^{2+} in this medium provides evidence that Ca^{2+} does not stimulate the exchanger directly. In P_i and citrate, Ca^{2+} is clearly unable to inhibit the K^+/H^+ exchanger, since Ca^{2+} addition resulted in rapid, electroneutral K^+ efflux (Fig. 6). This effect of Ca^{2+} loading may be of physiological significance. Recent work in Lehninger's laboratory has shown that mitochondria are capable of maintaining nearly constant external $[Ca^{2+}]$ following addition of Ca^{2+} or ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (14, 27). A sudden increase in cytosol Ca^{2+} will, therefore, result in rapid matrix swelling. In order to provide volume compensation, the K^+/H^+ exchanger must be capable of high rates of K^+ efflux. This capacity is suggested by the present data (Figs. 5C and 6) and is also consistent with our recent finding that the V_{max} of the exchanger is about 330 nmol of K^+ ($mg \cdot min$) $^{-1}$, far greater than the rate required to balance electrophoretic uptake of K^+ (24).

The present results should not be taken to imply that Ca^{2+} has no affinity for the inhibitory site on the K^+/H^+ exchanger. The dominant effect of Mg^{2+} in the normal, inhibited state can be attributed to the fact that its thermodynamic activity is sufficiently high to block the carrier. During Ca^{2+} uptake, matrix $[Ca^{2+}]$ may increase only slightly because of the attendant uptake of anions and water. These same factors will cause $[Mg^{2+}]$ to drop precipitously, and it is evidently this effect which predominates. To obtain information on the relative affinities of the K^+/H^+ exchanger for Ca^{2+} and Mg^{2+} , it is necessary to work at the left section of the titration curve in Fig. 7. It is in this region where we place the observation by Shi *et al.* (7) that Ca^{2+} is an effective blocker of K^+ efflux in mitochondria depleted of M^{2+} by pretreatment with A23187. We believe it is premature at this stage to draw conclusions from this finding, since Ca^{2+} , but not Mg^{2+} , had access to the matrix compartment under the conditions of the experiment. While knowledge of the relative affinities for Ca^{2+} and Mg^{2+} is desirable, the experimental approach is severely complicated by the difficulty in quantitating matrix activities of Ca^{2+} and Mg^{2+} and by the possibility of carrier asymmetry with respect to Mg^{2+} inhibition.

The present studies show that the inhibited K^+/H^+ exchanger can be turned on by Mg^{2+} depletion or by Ca^{2+} addition, but not by Ca^{2+} depletion. These results support the proposal that Mg^{2+} , not Ca^{2+} , plays the dominant physiological role in the K^+/H^+ carrier braking mechanism (5). In addition, these studies suggest a new homeostatic role for the

K^+/H^+ exchanger: to buffer against excessive matrix swelling secondary to Ca^{2+} uptake.

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