

## On the Mechanism of A23187-induced Potassium Efflux in Rat Liver Mitochondria\*

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1. Rat liver mitochondria undergo a spontaneous, respiration-dependent  $K^+$  extrusion which is accelerated by citrate. This  $K^+$  efflux is electroneutral and is considered to occur on an endogenous K/H exchanger. The spontaneous efflux, but not nigericin-induced K/H exchange, is always preceded by a lag phase, suggesting that the lag phase is a characteristic property of the endogenous exchange reaction.

2.  $K^+$  extrusion induced by ionophore A23187 also has the characteristics of K/H exchange. The rate of  $K^+$  efflux is faster and the lag time is shorter when compared to endogenous  $K^+$  efflux. The effects of A23187 on the lag phase suggest that the ionophore acts by unmasking the endogenous exchanger. This conclusion is supported by the finding that  $K^+$  efflux rates reach a maximum which cannot be exceeded by increasing the dose of A23187 but is exceeded by adding nigericin.

3. Steady state perturbation studies were carried out on respiring mitochondria in which electrophoretic  $K^+$  influx was balanced by electroneutral  $K^+$  efflux. These steady states were appropriately shifted in opposite directions by additions of nigericin or valinomycin. In contrast, addition of A23187 had no effect. It is concluded that A23187 is incapable of transporting  $K^+$  in rat liver mitochondria.

4. These results are consistent with a model in which free matrix  $Mg^{2+}$  acts as a K/H carrier "brake." The proposed role of this carrier-brake mechanism is to provide volume homeostasis with minimal energy expenditure. According to this model, both citrate and A23187 stimulate K/H exchange by reducing  $Mg^{2+}$  activity within the matrix. Citrate acts by complexation of  $Mg^{2+}$ , while A23187 acts by transporting  $Mg^{2+}$  out of the matrix.

The available evidence is consistent with Mitchell's postulate (1) that the mitochondrial membrane contains pathways for both electrophoretic and electroneutral  $K^+$  transport (2-11). These pathways have proved difficult to study independently, since their activities appear to move in parallel. Thus, the highest rates of  $^{42}K^+$  exchange occur under conditions favoring electrophoretic  $K^+$  movement, while inhibition of respiration results in very low rates of  $K^+$  turnover (2-6).

Hypotonic swelling at 0°C induces a net  $K^+$  efflux which is electroneutral and independent of respiration (10). High rates

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of  $K^+$  extrusion on a K/H exchanger were also observed in mitochondria respiring in tetraethylammonium salts (11). These experiments showed that K/H exchange could be separated experimentally from electrophoretic  $K^+$  transport and from respiration. For the first time, net  $K^+$  efflux, demonstrably electroneutral, was observed at rates up to  $70 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ , sufficient to account for reported  $^{42}K$  exchange rates (5, 6). The properties of this  $K^+$  efflux led Garlid to propose that the K/H exchanger is controlled by a matrix solute functioning as a "carrier brake" (10). The anion dependence of K/H exchange and the effects of  $Ca^{2+}$  led to the proposal that  $Mg^{2+}$  ions function as the carrier brake (12).

The divalent cation ionophore A23187 is known to deplete mitochondrial  $Mg^{2+}$  to very low levels (13-16). In principle, this ionophore should provide a dynamic means of investigating the proposed regulatory role of  $Mg^{2+}$  on K/H exchange. At the present time, however, such an approach is complicated by the fact that the mechanisms underlying A23187-induced  $K^+$  loss constitute an unresolved controversy in the literature. The extensive physicochemical studies by Pfeiffer and Lardy (17) led these authors to conclude that direct transport of  $K^+$  by A23187 was unlikely; however, their studies on intact mitochondria seemed to them to require that the ionophore did catalyze K/H exchange directly under some conditions. In contrast, Reed (18) concluded that the ionophore does not transport  $K^+$  across erythrocyte membranes, but that it increases  $K^+$  permeability by  $Ca^{2+}$ -dependent membrane alterations. Duszynski and Wojtczak (9) used albumin to deplete bound A23187 from liver mitochondria and concluded that A23187 was acting indirectly, via  $Mg^{2+}$  depletion, to release an endogenous  $K^+$  exchange mechanism.

We do not consider the conclusions in Ref. 9 to resolve this issue for two reasons: First, they are based on the assumption that a single wash removed the bound A23187. We consider it possible that the assay used to test this assumption (swelling in magnesium nitrate) is insufficiently sensitive, and the question of whether this ionophore can so readily be extracted from the mitochondrial membranes is addressed in a companion paper (19). Second, even if the conclusions in Ref. 9 are basically correct, the experiments reported therein are not capable of answering a question of critical importance raised by the work of Pfeiffer and Lardy (17): Does A23187 directly transport any  $K^+$  in mitochondria? If the answer is yes, then  $K^+$  kinetics in A23187-treated mitochondria is complex because they represent the sum of endogenous and exogenous carrier activities. On the other hand, if the answer is no, then this ionophore is an extraordinarily powerful tool for studies on  $K^+$  transport in mitochondria.

These considerations motivated us to determine the mechanism of A23187-induced  $K^+$  extrusion in rat liver mitochondria and, in particular, to determine whether this agent has

any ability to transport potassium in these mitochondria. The present studies on net  $K^+$  extrusion and on steady state  $K^+$  perturbations lead to the conclusion that A23187 does not transport  $K^+$ , but rather unmasks the endogenous K/H exchanger, presumably by depletion of divalent cations. A similar conclusion was drawn from a parallel study on beef heart mitochondria carried out at Ohio State University and presented in a companion paper (19). A preliminary report of these results has been presented (20).

#### EXPERIMENTAL PROCEDURES

**General Methodology**—Rat liver mitochondria were isolated using the high yield, differential centrifugation technique of Pedersen *et al.* (21). Stock suspensions containing 50 mg of protein  $ml^{-1}$  were stored in 0.25 M sucrose at 0°C. Aliquots (0.5 ml) were treated with rotenone ( $1 \mu g \text{ mg}^{-1}$ ) for 30 s, then added to 8 ml of media containing the  $K^+$  electrode.

Tetraethylammonium (TEA<sup>+</sup>) and trimethylamine salts were prepared by neutralization of the respective acids by the free base. Nigericin, valinomycin (Calbiochem), and A23187 (Calbiochem) solutions were gravimetrically prepared in dimethylsulfoxide. The nigericin used in these studies was the generous gift of Dr. W. E. Scott (Hoffman-LaRoche, Nutley, N.J.).

**Electrode Potential Measurements**—All measurements were carried out in a 25-ml polyallomer tube mounted in an insulated 5-liter container. Temperature was held constant at 25°C with a circulating water bath. The reaction vessel was fitted with an oxygen bubbling port and mounted on a submersible magnetic stirring unit. A monovalent cation electrode (Corning model 476220) coupled via a 0.15 M TEA/Cl salt bridge (Fisher Remote Reference Junction) to a calomel reference electrode (Corning model 476109) was used for all potential measurements. The depth of immersion of the electrodes was held constant. The reservoir containing the reference electrode was maintained at 25°C. Readings were obtained from an Orion Research Digital Analyzer pH meter (model 701A) interfaced with an Orion Printer (model 951) for data recording at 0.1-min intervals. A strip chart recorder was used to monitor the kinetics during the runs. In a typical run, the reaction vessel was placed in its housing, the electrodes submerged, and stirring and oxygenation begun. After 3 to 4 min of equilibration, the mitochondrial suspension was added.

For each medium the  $K^+$  electrode was calibrated by standard additions to a solution 8 ml of medium and 0.5 ml of 0.25 M sucrose. Nernstian behavior ( $[K]_0 = (A \cdot \exp [\alpha E])$ ) was observed and confirmed by atomic absorption spectroscopy.  $V \cdot \exp [\alpha E]$  was plotted versus added potassium (micromoles), where  $V$  is the total volume,  $E$  is the electrode potential in millivolts and  $\alpha = F/RT$ . The slope yields the constant  $A$ , characteristic for each medium, and the intercept corresponds to the amount of  $K^+$  initially present in the medium.

During each experiment, values of  $E(t)$  are printed at 0.1-min intervals. These values are put into a computer together with the parameters  $A$  and  $\alpha$ , and the value of  $[K]_0^i$  corresponding to each  $E(t)$  is computed. At the end of each run, Triton was added to a final concentration of 0.1% to solubilize the mitochondria and release matrix  $K^+$ . Protein was then determined by the biuret method (22) and potassium by atomic absorption spectroscopy or by standards additions to the solubilized sample with the electrodes in place. Total potassium ( $K_T$ ) and protein ( $P_T$ ) are constants for each run, while volume ( $V$ ) is a function of time due to additions made during the runs. These values are also put into the computer in order to obtain matrix potassium ( $\bar{K}_m$ ) at 0.1-min intervals, using the conservation relation

$$\bar{K}_m(t) = (K_T - V(t) \cdot (K)_0^i) / P_T$$

Individual data points are plotted in Fig. 2 to illustrate the results of this calculation. Since smooth curves fit the data to within 0.2  $nmol \cdot mg^{-1}$ , the remainder of the experiments are plotted without the data points for the purpose of clarity.

#### RESULTS

**The Effect of A23187 on Respiration-dependent Extrusion of  $K^+$** —In the presence of citrate, rat liver mitochondria respiring in 100 mM tetraethylammonium (TEA<sup>+</sup>) salts undergo a spontaneous extrusion of  $K^+$  (11). This reaction, shown in Curve *a* of Fig. 1, is characterized by a lag phase (1½ min) followed by a rapid efflux of  $K^+$  ( $25 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ ) until all the  $K^+$  is lost. The addition of valinomycin leads to reversal of the  $K^+$  efflux and uptake of  $K^+$  to a steady state level. This response to valinomycin establishes the electroneutrality of the efflux, characteristic of an obligatorily coupled K/H exchange mechanism (10).

Studies on the effects of A23187 were carried out under identical conditions to determine effects of A23187 on the characteristics of respiration-dependent K/H exchange. The addition of a moderate dose of A23187 ( $0.5 \text{ nmol} \cdot \text{mg}^{-1}$ ) at the time indicated led to a reduction in the lag time and an increase in the maximum rate of  $K^+$  efflux to  $100 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$  (Fig. 1, Curve *b*). Increasing the dose of A23187 further reduced the duration of the lag phase and increased the maximal rate of efflux (not shown). The qualitative characteristics of endogenous K/H exchange, a lag phase followed by a rapid and complete  $K^+$  extrusion, were not effected by A23187. However, there was a dose-dependent effect of A23187 on the duration of the lag phase and on the maximum rate of  $K^+$  efflux.

In the  $Mg^{2+}$  carrier-brake model (12), the lag phase reflects the time required for reduction of free matrix  $Mg^{2+}$ , by complexation with citrate, to levels below that required to inhibit the K/H exchanger. Since A23187 causes ejection of  $Mg^{2+}$  from the matrix, the present data are consistent with the carrier-brake model. Furthermore, the inability of A23187 to abolish the lag phase is suggestive of an indirect effect of this ionophore on K/H exchange. The possibility exists, however, that divalent cations have precedence over  $K^+$  due to their higher affinities for the ionophore, as suggested by Pfeiffer

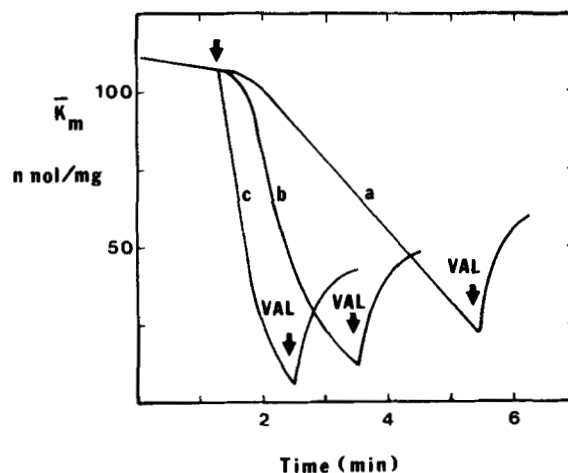


FIG. 1. The effects of A23187 and nigericin on mitochondrial  $K^+$ . Following pretreatment with rotenone ( $1 \mu g \cdot \text{mg}^{-1}$ ), mitochondria were suspended in a medium containing  $K^+$  (0.25 mM) and TEA<sup>+</sup> salts of succinate (11 mM), phosphate (1 mM), malate (1 mM), TES (pH 7.2, 11.5 mM), citrate (6.5 mM), EGTA (0.05 mM), and EDTA (0.9 mM) made isotonic (0.272 osmolal) with sucrose. Values for mitochondrial  $K^+$  (see "Experimental Procedures") are plotted versus incubation time for three separate experimental runs. No ionophore was added at the start of Run *a*; at the first arrow 0.5  $nmol \cdot \text{mg}^{-1}$  A23187 was added in Run *b* and 3.0  $pmol \cdot \text{mg}^{-1}$  nigericin was added in Run *c*. Controls were unaffected by dimethylsulfoxide, used as a solvent for the ionophores, when added to final concentrations up to 0.1 M. Near the end of  $K^+$  efflux, 0.5  $nmol \cdot \text{mg}^{-1}$  valinomycin was added to each run.

<sup>1</sup> The abbreviations used are: TEA<sup>+</sup>, tetraethylammonium ion; TMAM, trimethylamine; VAL, valinomycin; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether) *N,N,N',N'*-tetraacetate;  $[K]_0$ , potassium concentration in the suspending medium (mM);  $\bar{K}_m$ , matrix potassium ( $nmol \cdot \text{mg}^{-1}$ ).

and Lardy (17). This possibility was tested by adding A23187 at the time ( $\pm 5$  s) when endogenous  $K^+$  efflux would normally begin. Under these conditions there was no lag phase, and the rate induced by A23187 was immediately greater than the endogenous rate (not shown). If the faster rate is due to A23187 acting as a  $K^+$  ionophore, these results appear to require that divalent cations do not take precedence over  $K^+$ .

**Nigericin-induced  $K^+$  Extrusion**—The lag phase was expected to be a consequence of the carrier-brake and not of the K/H exchange itself. To test this hypothesis, nigericin ( $1.0 \text{ pmol} \cdot \text{mg}^{-1}$ ) was added to mitochondria respiring in media identical with the previous studies. Indeed, no lag phase was observed with nigericin (Fig. 1, Curve C) adding support to the contention that A23187 induces  $K^+$  loss indirectly, rather than directly.

**The Effects of Anions on A23187-induced  $K^+$  Extrusion**—The anion composition of the medium has a profound effect on endogenous K/H exchange. Rotenone-treated mitochondria utilizing succinate as a substrate exhibit very slow K/H exchange following a long lag time (Fig. 2, Curve A). According to the carrier-brake model, these effects are due to the paucity of Mg-chelating anions in the matrix (12). Addition of A23187 to these mitochondria resulted in a reduction in lag phase and an increase in the rate of K/H exchange (Fig. 2, Curve b). In parallel with the effect of citrate on the spontaneous reaction, the lag phase of A23187-induced  $K^+$  efflux is of longer duration in the absence of citrate (compare Fig. 1 with fig. 2).

**Dose-response Studies on  $K^+$  Efflux Induced by A23187**—If A23187 transports  $K^+$ , the rate of K/H exchange should increase with the dose of the ionophore. A23187 was added at the end of the endogenous lag phase and the dose-response curves are plotted in Fig. 3. At a dose of  $1.0 \text{ nmol} \cdot \text{mg}^{-1}$  A23187 in  $134 \text{ mM TEA}^+$ , the rate of  $K^+$  extrusion was  $150 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ . This rate could not be exceeded with larger doses of A23187, up to  $15 \text{ nmol} \cdot \text{mg}^{-1}$ .

Taken alone, this result strongly supports the contention that A23187 does not transport  $K^+$ . If A23187 is a potassium carrier, the rate of  $K^+$  efflux should increase with increasing concentration of added carrier. However, we also carried out experiments at various  $\text{TEA}^+$  concentrations (Fig. 3) and found that maximal rates of  $K^+$  extrusion depend on the concentration of  $\text{TEA}^+$  as well as that of A23187. This result casts doubt on the initial conclusion, since it raises the possibility that the rate limitations are due to limits on the gradients of  $K^+$  and  $H^+$  rather than limits on their carrier-

mediated permeabilities. To test this possibility, we next carried out dose-response studies on mitochondria treated with A23187 and with nigericin in identical media (Fig. 4). It can be seen that the nigericin-induced  $K^+$  extrusion was dose-dependent and occurred at rates in excess of the maximal rate observed with A23187. That is, the maximal rate of  $K^+$  efflux is not gradient-limited, but rather is limited by the amount of K/H exchanger available.

**The Dependence of  $K^+$  Extrusion on External  $\text{Mg}^{2+}$** —It was considered important to establish that external  $\text{Mg}^{2+}$  inhibits A23187-induced  $K^+$  efflux, since such an inhibition is predicted by both mechanisms. If A23187 is transporting  $K^+$ , there should be a prominent inhibition of  $K^+$  efflux by  $\text{Mg}^{2+}$  due to its much higher affinity for the ionophore (17). If  $K^+$  efflux is stimulated secondary to  $\text{Mg}^{2+}$  ejection from the matrix (9), it should likewise be inhibited by external  $\text{Mg}^{2+}$  as a result of lowering the driving force for  $\text{Mg}^{2+}$  ejection. Inhibition by  $\text{Mg}^{2+}$  was observed in experiments in which  $\text{Ca}^{2+}$  was trapped by EGTA, and the degree of inhibition was found to depend on the dose of A23187 (Fig. 5). We did not consider it productive to attempt to distinguish between the two mechanisms by kinetic arguments, since the kinetics is complicated by the association of 2 molecules of A23187/ $\text{Mg}^{2+}$  ion (13, 17).

**Steady State Perturbation Studies**—Addition of low doses of valinomycin to mitochondria respiring in  $\text{TEA}^+$  salts resulted in an initial uptake of  $K^+$  followed by a spontaneous, gradual loss of  $K^+$  (Fig. 6, dashed line). The  $K^+$  loss was accelerated by low doses of A23187, resulting in a constant, steady state level of  $K^+$  (Curve b).

The framework within which these studies were carried out is conceptually simple. Net  $K^+$  transport is the vectorial sum of two processes: The electrophoretic flux,  $J_{K^+}$ , depends on the electrochemical potential gradient for  $K^+$  and on the concentration of valinomycin added to the system. The electroneutral flux,  $J_{K^0}$ , depends on the chemical gradients of  $K^+$  and  $H^+$  and upon the turnover rate of the endogenous exchanger under the conditions of observation. Electroneutral flux on putative exogenous K/H exchangers, such as nigericin and A23187, must be dose-dependent. Net  $K^+$  flux,  $J_K$ , can be written

$$J_{K^+} + J_{K^0} = J_K \quad (1)$$

When mitochondrial potassium is constant ( $J_K = 0$ ), the addition of more valinomycin will result in a net  $K^+$  flux if, and only if, the previous state was a steady state. That is,

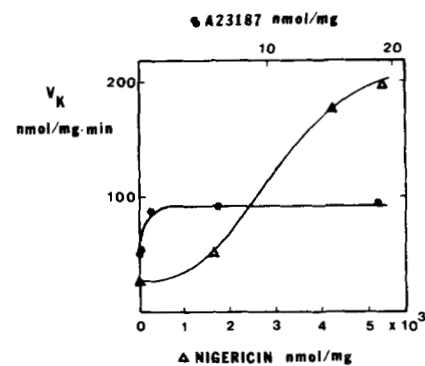
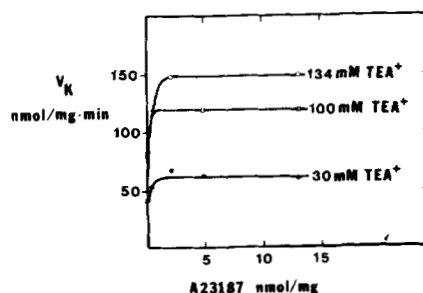
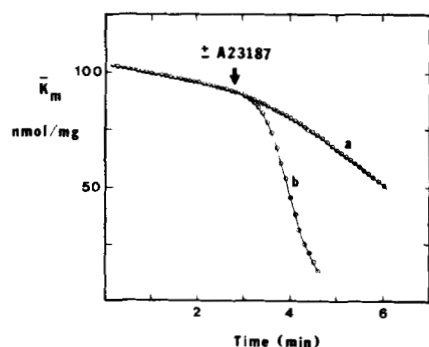


FIG. 2 (left). A23187-induced  $K^+$  extrusion in the absence of citrate. The experimental conditions were as described in Fig. 1 except that citrate was omitted from the medium. No ionophore was added in Run a; in Run b, the A23187 dosage used was  $2.5 \text{ nmol} \cdot \text{mg}^{-1}$ . Individual data points are also plotted for the purpose of demonstrating the basis of the experimental curves (see "Experimental Procedures").

FIG. 3 (center). Dose-response studies of  $K^+$  efflux induced by A23187. The experimental conditions were as described in Fig. 2.

TEA<sup>+</sup> concentration was varied by substituting 0.25 M sucrose for 0.15 M TEA Cl in making up the stock salt solutions. A23187 dosages ranged from 0 to  $13.2 \text{ nmol} \cdot \text{mg}^{-1}$  protein. The data plotted represent the maximum efflux rate,  $V_K$ , observed with the A23187 dosage at the given TEA<sup>+</sup> level.

FIG. 4 (right). A comparison of the effects of nigericin and A23187 on  $K^+$  efflux. The experimental conditions were as described in Fig. 1.

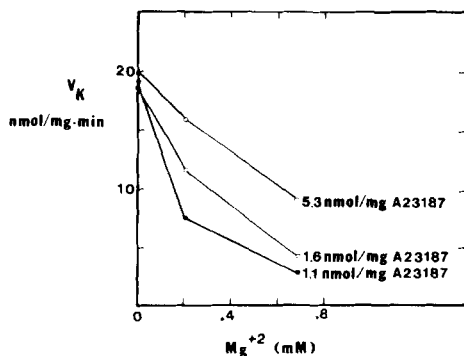
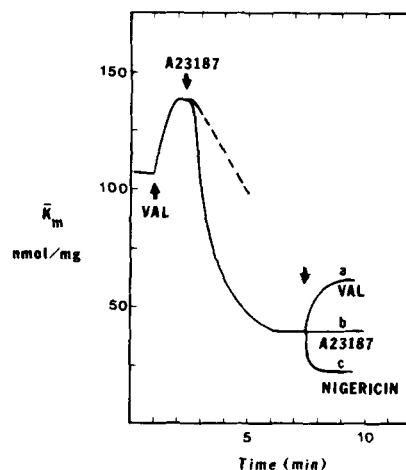


FIG. 5 (left). The effect of external  $Mg^{2+}$  on A23187-induced  $K^+$  efflux. Rotenone-treated mitochondria were suspended in a medium containing sucrose (0.2 M) and  $TEA^+$  salts of TES (20 mM), acetate (15 mM), and EGTA (0.1 mM) to a final concentration of 5  $mg \cdot ml^{-1}$  protein. Varying amounts of  $Mg^{2+}$  were added and concentrations reflect total  $Mg^{2+}$  found by atomic absorption spectroscopy. Addition of 1.0 mM EDTA instead of  $Mg^{2+}$  was taken to represent zero  $Mg^{2+}$ . One minute after the indicated doses of A23187 were added, respiration and  $K^+$  efflux were initiated by the addition of TEA succinate (6.25 mM).

FIG. 6 (right). Steady state perturbation studies in the absence of citrate. The experimental conditions were as described for Fig. 2. An initial low dose of valinomycin (5  $pmol \cdot mg^{-1}$ ) was followed by no addition (dashed line) or by a low priming dose of A23187 (0.3  $nmol \cdot mg^{-1}$ , solid line). In separate runs, further additions were made after the steady state was achieved at the time indicated by the arrow. Run a, valinomycin, 1  $nmol \cdot mg^{-1}$ ; Run b, no addition or A23187 in doses ranging from 1 to 10  $nmol \cdot mg^{-1}$ ; Run c, nigericin, 1  $pmol \cdot mg^{-1}$ . This figure summarizes the results of over 40 such runs carried out on five separate preparations. When conditions were constant, absolute values of  $\bar{K}_m$  from separate runs carried out on the



same day were reproducible to within  $\pm 5$   $nmol \cdot mg^{-1}$ . Day-to-day variations were greater, but the patterns were identical with those shown. Numerous additional studies were carried out to characterize these effects further, and the results can best be described in the context of this figure. Repeat additions of valinomycin at the plateau of Curve a had no effect, a finding which implies that 1  $nmol \cdot mg^{-1}$  is sufficient to induce electrochemical equilibrium for  $K^+$ . The response to valinomycin addition at the arrow (Curve a) was completely unaffected by 10  $nmol \cdot mg^{-1}$  A23187, whether added prior to or after the valinomycin. The plateau observed after nigericin addition (Curve c) was likewise unaffected in rate of approach or magnitude by 10  $nmol \cdot mg^{-1}$  A23187, whether added prior to or after the nigericin. Conversely, raising the dose of nigericin added at the arrow resulted in a lower steady state  $\bar{K}_m$  until all  $K^+$  was lost. In another series, the time of addition of the initial priming dose of A23187 was varied from 30 s to 8 min, valinomycin being added at 1 min. In all cases,  $\bar{K}_m$  declined to the same steady state level (Curve b) as that shown in the figure. When high doses of valinomycin (1  $nmol \cdot mg^{-1}$ ) and A23187 (5  $nmol \cdot mg^{-1}$ ) were added at the start of the incubations,  $\bar{K}_m$  declined to the same equilibrium level (Curve a) as that shown in the figure. The magnitude of this plateau was unaffected by the order of addition.

steady state  $K^+$  is the result of equal and opposing electrophoretic and electroneutral fluxes. High levels of valinomycin should be capable of inducing electrophoretic equilibrium of  $K^+$  due to "swamping out" of the neutral exchanger ( $J_{K^+} \gg J_K^0$ ). This effect is seen in Fig. 6, Curve a. Further additions of valinomycin had no effect on  $K^+$  (not shown). The steady state was also perturbed by low doses of nigericin, which shifts the balance in favor of  $J_K^0$ , leading to an efflux of  $K^+$  (Fig. 6, Curve c). Large doses of nigericin result in complete loss of  $K^+$ , representing equilibrium of  $K^+$  with respect to K/H exchange.

From these results it is evident that there is an underlying K/H exchange at the steady state. The addition of A23187 (1 to 5  $nmol \cdot mg^{-1}$ ) at this point was incapable of causing  $K^+$  efflux (Fig. 6, Curve b). This leaves the possibility that gradients, rather than permeabilities, were limiting K/H exchange; however, nigericin addition caused a rapid shift to a new steady state at a lower level of  $K^+$  (Fig. 6, Curve c). Furthermore, the steady state perturbations by nigericin and valinomycin were unaffected in magnitude or direction by the prior addition of A23187, at any dose, during the steady state. We conclude from these results that A23187 does not function as a K/H exchanger.

This experiment is particularly persuasive in view of the excess EGTA and EDTA present in the medium. These chelators have high affinities for  $Mg^{2+}$  and  $Ca^{2+}$ , so that most of the A23187 must be present in the free acid form. This condition, together with the ongoing respiration, should be most favorable for the complexation with  $K^+$  postulated by Pfeiffer and Lardy (17); nevertheless there is no perturbation

whatsoever of the steady state upon addition of A23187.

$K^+$  Perturbation in  $TEA^+$  Citrate—The endogenous carrier is capable of operating at rapid rates in the presence of citrate (Fig. 1). It seemed that it might be possible to induce steady state  $K^+$  with valinomycin alone, without the priming dose of A23187. This proved to be the case, as seen in Fig. 7. In this experiment, addition of valinomycin resulted in an initial uptake of  $K^+$  followed by a spontaneous, rapid  $K^+$  efflux to a new steady state level. When A23187 was added during the efflux phase, there was a transient uptake, rather than efflux, of  $K^+$  followed by a rapid decay to a level indistinguishable from that observed when no A23187 was added. This transient was only observed following the first addition of A23187; subsequent additions had no effect whatsoever on  $K^+$  kinetics.

$K^+$  uptake is remarkable considering the fact that it occurred when matrix  $K^+$  was 40 to 120  $nmol \cdot mg^{-1}$ , while external  $[K^+]$  was 0.2 to 0.3 mM. This phenomenon can readily be understood in terms of the chemiosmotic hypothesis (1): The protonmotive force is the sum of  $\Delta pH$  and electrical potential ( $\Psi$ ) terms. Addition of a large dose of A23187 results in a rapid influx of protons (in exchange for  $Mg^{2+}$  and  $Ca^{2+}$ ) which is transiently uncompensated by the slower efflux of citrate, leading to a drop in  $\Delta pH$ . This drop must necessarily be compensated by an increase in the magnitude of  $\psi$  and, hence, in the electrochemical gradient for  $K^+$ , leading to  $K^+$  uptake until anions return to equilibrium. This interpretation of the transient is consistent with the finding with safranin dye that an increase in potential gradient accompanies the addition of A23187 (19). As in the previous study, if A23187

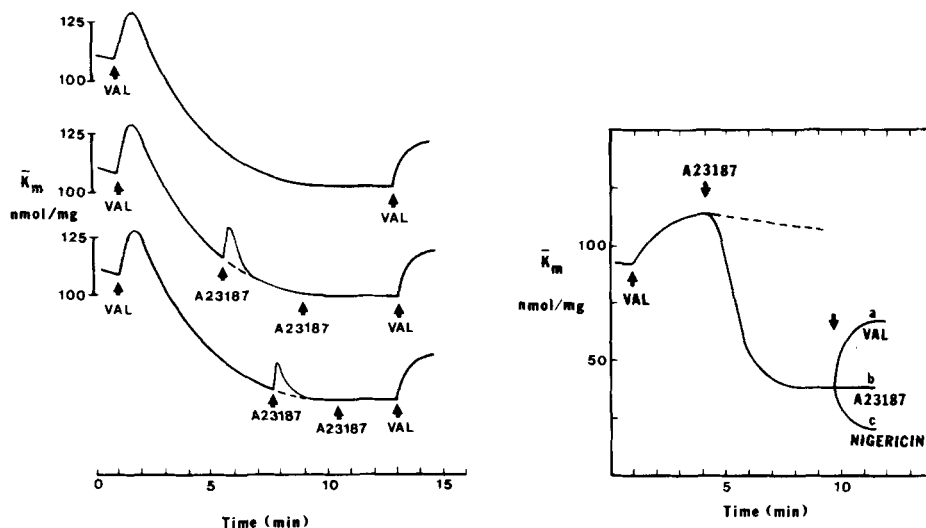


FIG. 7 (left). **Steady state perturbation studies in media containing citrate.** The experimental conditions were as described for Fig. 6 except that citrate (8.3 mM) was added. In this medium, mitochondria lose all of their K<sup>+</sup> spontaneously within 6 min (see Fig. 1, Curve a). A low dose of valinomycin (5 pmol·mg<sup>-1</sup>) induces K<sup>+</sup> uptake followed by a spontaneous efflux to a steady state (Curve a) which is shifted maximally toward equilibrium by adding an additional 1 nmol·mg<sup>-1</sup> dose of valinomycin. Curves b and c show the effects of A23187 (3 nmol·mg<sup>-1</sup>), added at different times, on this pattern of K<sup>+</sup> efflux. The dashed lines represent the values from the control run, Curve a. Note that a second addition of A23187 following the transient had no effect.

FIG. 8 (right). **Steady state perturbation studies in trimeth-**

ylamine salts. Mitochondria were pretreated with rotenone (1 μg·mg<sup>-1</sup>) and suspended in a medium containing K<sup>+</sup> (0.25 mM) and TMAM salts of malate (0.5 mM), phosphate (0.5 mM), EDTA (0.4 mM), EGTA (0.04 mM), TES (pH 7.2, 11.5 mM), and succinate (5 mM) made isotonic (0.272 osmolal) with sucrose. The experimental protocol was identical with that described for Fig. 6: An initial low dose of valinomycin (5 pmol·mg<sup>-1</sup>) was followed by no addition (dashed line) or by a low priming dose of A23187 (0.3 nmol·mg<sup>-1</sup>, solid line). In separate runs, further additions were made after the steady state was achieved at the time indicated by the arrow. Run a, valinomycin, 1 nmol·mg<sup>-1</sup>; Run b, no addition or 10 nmol·mg<sup>-1</sup> A23187; Run c, nigericin, 1 pmol·mg<sup>-1</sup>. The ancillary studies described in the legend to Fig. 6 were also carried out in TMAM salts with the same results.

had been transporting K<sup>+</sup> directly, a more rapid loss of K<sup>+</sup> to a lower steady state would have followed the transient.

In the studies summarized in Fig. 6, a priming dose of A23187 was required for the rapid achievement of a steady state. It is noteworthy that the addition of citrate removes this requirement, supporting the contention that release of K/H exchange requires removal of divalent cations rather than the presence of A23187.

**Steady State Perturbations in Trimethylamine Salts—**While the previous data on steady state perturbations appear to confirm the contention that A23187 does not transport K<sup>+</sup> directly, it was considered desirable to go to a system in which there was also a volume steady state. For this purpose we carried out the remaining experiments using trimethylamine (TMAM). TMAM crosses the membrane largely by nonionic diffusion, rather than by electrophoretic diffusion as is the case with the quaternary ammonium ion, TEA<sup>+</sup>. Respiration in TMAM is therefore associated with a steady state volume rather than the swelling which occurs when TEA<sup>+</sup> uptake is not balanced by K<sup>+</sup> efflux.

In Fig. 8 are plotted the results of several experiments carried out in TMAM salts. A steady state of long duration is achieved following treatment with a low dose of valinomycin (5 pmol·mg<sup>-1</sup>, dashed line). The steady state is shifted downward after treatment with a low dose of A23187 (0.3 nmol·mg<sup>-1</sup>, solid line). The result of subsequent additions of valinomycin (uptake) or nigericin (efflux) demonstrate that this new level is a true steady state and that an underlying K/H exchange is taking place. Once again, addition of A23187 to this steady state has no effect, confirming that A23187 is releasing an endogenous exchanger and does not transport K<sup>+</sup> itself.

#### DISCUSSION

The present studies confirm the original findings of Reed

and Lardy (13) that the ionophore A23187 induces mitochondria to extrude K<sup>+</sup> as well as Ca<sup>2+</sup> and Mg<sup>2+</sup>. K<sup>+</sup> efflux is increased by A23187, even when the underlying endogenous exchanger is already operating at high rates (Figs. 1 and 2). The characteristics of the stimulated efflux resemble those of the endogenous exchanger, rather than those of an exogenous K/H exchanger such as nigericin. Thus, nigericin-induced K<sup>+</sup> efflux is not stimulated by citrate, nor is it associated with a lag phase, in contrast to endogenous and A23187-induced efflux (Fig. 1). These results suggest that A23187 releases an endogenous K/H exchanger, as proposed by Duszynski and Wojtczak (9). While the experiments reported in (9) are consistent with this conclusion, they do not rule out an additive contribution of A23187 to K/H exchange. That is, they do not directly address the question of whether A23187 is capable of transporting K<sup>+</sup>, as was concluded by Pfeiffer and Lardy (17). The importance of this question may be seen by considering the A23187-induced K/H exchange rate of 150 nmol·mg<sup>-1</sup>·min<sup>-1</sup> (Fig. 3). Whether or not this rate solely reflects the capacity of the endogenous K/H exchanger is clearly significant for our understanding of K<sup>+</sup> homeostasis and energy conservation in mitochondria.

Two findings reported here do resolve this question: 1) A23187-induced K<sup>+</sup> efflux reaches a maximum rate which cannot be exceeded by increasing the amount of ionophore. Under the same conditions, this rate is readily exceeded by nigericin (Figs. 3 and 4). 2) Steady state perturbation studies demonstrate the existence of an underlying K/H exchange during respiration. Under several different conditions, the steady state is perturbed in the appropriate direction by valinomycin or nigericin, but is unaffected by A23187 at any dose (Figs. 6 to 8). We consider these findings to establish conclusively that A23187 does not transport K<sup>+</sup> in rat liver mitochondria.

While Pfeiffer and Lardy (17) came to the opposite conclu-

sion, they did so with strong reservations. For example, they found that extraction of  $K^+$  with A23187 into hydrophobic solvents was only possible in the strict absence of divalent cations and required a pH of 9.0. Even under these conditions, the extraction was not very efficient. These authors noted that this finding made it unlikely that the ionophore could transport  $K^+$  in biological membranes. The present study confirms this expectation.

We have presented evidence that A23187 does not transport  $K^+$ ; we must next address the question of how A23187 stimulates K/H exchange. This requires an understanding of the endogenous mechanism for K/H exchange in mitochondria, a matter which is still under active investigation. Nevertheless, it is necessary to use some model as a framework for explaining the actions of A23187, and the  $Mg^{2+}$  carrier-brake hypothesis (12) has the advantage of providing a rather specific underlying mechanism which is consistent with experimental observations (10–12). We emphasize that the ensuing discussion must be considered conjectural at this time, to the extent that it is based on a model which has not yet been established.

In the carrier-brake model, reversible binding of  $Mg^{2+}$  to the K/H exchanger is considered to inhibit K/H exchange and thereby provide a finely tuned response to the electrophoretic uptake of cations. The Mg-carrier equilibrium is dynamically modified by the uptake of Mg-complexing anions which inevitably accompany cation uptake. Thus, small changes in total anions result in changes in free matrix  $Mg^{2+}$  concentration, shifting the Mg-carrier equilibrium in the appropriate direction.  $Mg^{2+}$  is well suited for the role of carrier brake because its rate of transport is slow by comparison with  $K^+$  and organic anions (23–25). The result of this mechanism is that energy is conserved, only that amount of  $K^+$  is ejected on the exchanger which is required to balance the extra  $K^+$  taken up electrophoretically. Thus, futile cycling of  $K^+$  and  $H^+$  is prevented by the carrier-brake.

Mitochondria swell during respiration in tetraethylammonium salts, secondary to the electrophoretic uptake of  $TEA^+$  and electroneutral uptake of anions to compensate the proton ejection (11). According to the model, the lag phase of  $K^+$  efflux represents the time required for swelling and complexation to titrate  $Mg^{2+}$  off the carrier. Thus, the lag phase is greatly attenuated in the presence of citrate (Fig. 1 versus Fig. 2). Whatever the endogenous lag time, it is reduced by A23187-induced depletion of  $Mg^{2+}$ . When time is allowed for complexation of matrix  $Mg^{2+}$  by citrate, addition of A23187 induces an immediate increase in  $K^+$  efflux by further reducing matrix  $Mg^{2+}$ , freeing up additional carrier. According to the model, the lag time required for A23187-induced  $K^+$  efflux is increased in the presence of external  $Mg^{2+}$ . Inhibition of A23187-induced  $K^+$  efflux by external  $Mg^{2+}$  (Fig. 5) can be explained in the same manner.

The observation that  $TEA^+$  concentration determines the maximal rate of A23187-induced potassium efflux (Fig. 3) requires a more complex explanation. We have observed that endogenous K/H exchange declines rapidly as the pH falls below 7.2<sup>2</sup> and a similar pH dependency has been observed in studies on the Na/H exchange reaction (26). We speculate that this inherent pH sensitivity is responsible for the dependence of  $V_{max}$  on  $TEA^+$  concentration. Proton ejection by the respiratory assembly depends on the  $TEA^+$  influx rate, and hence on external  $[TEA^+]$ . When K/H exchange equals  $TEA^+$  uptake, there will be a balance of proton transport and a cessation of net anion uptake. Free matrix  $Mg^{2+}$  will remain constant at a level determined by the availability and affinities of Mg-complexing moieties, resulting in a constant rate of  $K^+$  efflux. By removing  $Mg^{2+}$  from the matrix, A23187 is able to

stimulate this efflux, resulting in an imbalance between  $K^+$  and  $TEA^+$ , acidification of the matrix, and partial inhibition of the exchanger. Nigericin-induced cation exchange is also known to be inhibited at acid pH levels (27); nevertheless, this inhibition can be overcome by increasing the dose of the ionophore, while the amount of endogenous exchanger is fixed. This explains the finding that nigericin-induced K/H exchange can be made to exceed A23187-induced exchange under identical conditions (Fig. 4).

The mechanism of action of A23187 is therefore considered to be depletion of  $Mg^{2+}$  ions from the matrix, thereby unmasking the endogenous K/H exchanger. This explanation is in agreement with that offered by Duszynski and Wojtczak (9) and consistent with the previously reported effects of citrate (11) and of swelling (10). The present results are consistent with the proposed role for  $Mg^{2+}$  in regulating the K/H exchanger; however, their design prohibits an evaluation of a possible regulatory role for  $Ca^{2+}$ . Thus, in the presence of A23187,  $Ca^{2+}$  must be trapped with EGTA to prevent rapid  $Ca^{2+}$  cycling and uncoupling. The effects of  $Ca^{2+}$  on albumin-washed preparations are presented in a companion paper (19).

Finally, it is noteworthy that the endogenous exchanger is capable of transporting  $K^+$  at a rate of  $150 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$  (Fig. 3). Whatever the precise nature of carrier regulation, the need for an inhibiting mechanism to prevent futile cycling of  $K^+$  and  $H^+$  is apparent. While K/H exchange is necessary for volume homeostasis (4, 10), precise regulation of this exchange is essential for energy conservation (11, 12).

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