Regulation of the Mitochondrial Ca^{2+} Uniporter by External Adenine Nucleotides: The Uniporter Behaves Like a Gated Channel Which Is Regulated by Nucleotides and Divalent Cations†

Monica L. Litsky and Douglas R. Pfeiffer*

Department of Medical Biochemistry, College of Medicine, The Ohio State University, Columbus, Ohio 43210

Received January 27, 1997; Revised Manuscript Received April 9, 1997⊗

ABSTRACT: We have previously used measurements of uncoupler-enforced reverse activity to demonstrate that the mitochondrial Ca^{2+} uniporter is strongly inhibited by external EGTA plus free Mg^{2+}, following a brief period of rapid activity. Using the same approach, we now show that in addition to divalent cations, the uniporter is regulated by external adenine nucleotides and by other components of the cytosol. Inhibition produced by EGTA plus free Mg^{2+} is reversed by spermine ($EC_{0.5} \approx 40$ μM) and reduced when mitochondria are purified by an isoosmotic density-gradient method. Under either condition, inhibition is restored by external adenine nucleotides in a concentration-dependent manner. The order of effectiveness is ATP > ADP > AMP, with the nucleoside adenosine being ineffective. Among nucleotide triphosphates, the order is ATP > CTP ≈ UTP > GTP. The effectiveness of ATP ($EC_{50} \approx 0.6$ mM) is the same in mitochondria and mitoplasts, the same as that of AMPPNP, and is not altered by the presence of oligomycin, carboxyatractyloside, or AP_5A, used alone or in combinations. These findings indicate that ATP acts at a site located on the outer surface of the inner membrane through a mechanism which does not require its hydrolysis. Phosphate also inhibits reverse uniport under some conditions ($EC_{50} \approx 20$ μM). The sites at which free ATP and free Mg^{2+} inhibit the uniporter can be distinguished by chymotrypsin treatment of mitoplasts, which eliminates the action of Mg^{2+} but does not affect the action of ATP. Data are interpreted within the context of a model in which the uniporter is considered to be a gated channel that is controlled, in part, by specific external effector sites that accept divalent cations or nucleotides. The possible consequences of the model for cell Ca^{2+} regulation by mitochondria and regulation of TCA cycle activity by the matrix free Ca^{2+} concentration are considered.

Mitochondria from all vertebrate and from many invertebrate sources accumulate and release Ca^{2+} through the action of specific inner membrane proteins. Ca^{2+} accumulation occurs via the ruthenium red-sensitive Ca^{2+} uniporter, which allows a charge-uncompensated movement of Ca^{2+} down its electrochemical gradient (Gunter & Pfeiffer, 1990; Gunter et al., 1994). The uniporter is thought to be an ion-conducting channel, based upon its apparent turnover number and voltage dependence (Gunter & Pfeiffer, 1990; Sparagna et al., 1995). Release of Ca^{2+} occurs via several activities with the best characterized being the Na^+/Ca^{2+} antiporter. This transporter normally exchanges matrix Ca^{2+} for external Na^+ and was originally thought to function by an electroneutral mechanism (i.e., 2 Na^+ for Ca^{2+} exchange) (Brand, 1985). However, recent work indicates a higher stoichiometry with respect to Na^+, and that the reaction is, in fact, electrogenic (Baysal et al., 1994; Jung et al., 1995). Mitochondria also release Ca^{2+} by a Na^+-independent mechanism which is energy-requiring (Gunter et al., 1983), and perhaps through forms of the permeability transition pore (Kass et al., 1992; Altschuld et al., 1992).

Cyclic accumulation and release of Ca^{2+} occur through the actions of the Ca^{2+} uniporter and the opposing transporters, such that the concentration of free Ca^{2+} within the matrix space is precisely regulated. The matrix Ca^{2+} concentration is thought to be subject to extensive regulation, in part, because Ca^{2+} participates in the control of NADH production by the TCA cycle and hence in the regulation of ATP synthesis by mitochondria (McCormack et al., 1990; Hansford, 1994). In addition to controlling NADH generation, matrix Ca^{2+} may also influence the rate of ATP synthesis through actions exerted at the levels of electron transport (e.g., McCormack et al., 1990; Kotlyar et al., 1992; Panov & Scaduto, 1995), the adenine nucleotide translocase (e.g., Moreno-Sánchez, 1985; Halestrap, 1987; Brown, 1992), and the ATP synthase *per se* (e.g., Yamada & Huzel, 1989; Harris & Das, 1991; Bogucka et al., 1995; Panov & Scaduto, 1995), at least under some conditions.

The mitochondrial Ca^{2+} cycle has additional functions which include defending cells against large and sustained transients in cytoplasmic Ca^{2+} concentration brought on by limited periods of ischemia or other injurious conditions (Farber, 1990; Silverman, 1993; Ferrari et al., 1993; Allen et al., 1993; Kiedrowski & Costa, 1995; Miller & Tormey, 1995). During the acute phase, the high-activity uniporter allows mitochondria to accumulate Ca^{2+} rapidly, limiting the deleterious effects of a high cytoplasmic concentration. Upon removal of the insult, the sequestered Ca^{2+} can be released in a controlled fashion, and distributed by the extramitochondrial Ca^{2+} transporters, while the cyto-

† This research was supported by U.S. Public Health Service Grant HL 49182 from the National Institutes of Health, National Heart, Lung, and Blood Institute.

* Address correspondence to this author at the Department of Medical Biochemistry, The Ohio State University, 1645 Neil Ave., 310A Hamilton Hall, Columbus, OH 43210-1218. Phone: 614-292-8774. Fax: 614-292-4118. E-mail: pfeiffer.17@postbox.acs.ohio-state.edu.

⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1997.

S0006-2960(97)00180-3 CCC: $14.00

plasmic concentration is maintained below the threshold of toxicity.

Recent studies indicate that the mitochondrial Ca^{2+} cycle also participates in regulation of cell Ca^{2+} levels by signaling mechanisms under physiological conditions. This concept is actually an old idea which was once widely held, but lost favor when it was determined that isolated mitochondria buffer external free Ca^{2+} at levels above those found in unstimulated cells (Carafoli, 1987). However, recent studies conducted with engineered cell lines that express aequorin in mitochondria have shown that mitochondrial Ca^{2+} transport limits and extends the rise in cytoplasmic Ca^{2+} concentration produced by cell signaling mechanisms (Rizzuto et al., 1992, 1993, 1994; Rutter et al., 1993). This action was first thought to be significant primarily within subcellular regions located near Ca^{2+}-conducting channels in nonmitochondrial membranes, where the local Ca^{2+} concentration would be particularly high during cell stimulation (Rizzuto et al., 1992, 1993, 1994; Rutter et al., 1993; Lawrie et al., 1996). However, it is now clear that in some cells, mitochondria dominate in clearing an excitatory Ca^{2+} load, even when considered at the whole cell level (Park et al., 1996; Herrington et al., 1996; Babcock et al., 1997). Additional reports based upon the application of several methodologies also show that mitochondrial Ca^{2+} transport is quantitatively important in regulating the cytoplasmic as well as the matrix Ca^{2+} concentration (e.g., Loew et al., 1994; Werth & Thayer, 1994; White & Reynolds, 1995; Drummond & Fay, 1996). These include the important demonstration that in *Xenopus* oocytes, the form and periodicity of cytoplasmic Ca^{2+} waves are greatly altered when the mitochondrial Ca^{2+} cycle is interrupted (Jouaville et al., 1995).

Recognition that the mitochondrial Ca^{2+} cycle plays several roles in physiological and pathological processes has renewed interest in the physical properties of the transporters which are involved, and in investigating the control of their activities. The present report addresses the latter subject with a focus upon regulation of the Ca^{2+} uniporter. By studying the activity of this transporter when it is operating in reverse (i.e., cation release), we show that external adenine nucleotides have strong regulatory effects. The data are interpreted within the context of a model wherein the Ca^{2+} uniporter is considered to be a gated channel which is regulated by mechanisms that are analogous to those which control channels in other cellular membranes. Aspects of these findings have appeared in abstract form (Litsky & Pfeiffer, 1995, 1996).

EXPERIMENTAL PROCEDURES

Reagents. Common chemicals were obtained from commercial sources and were reagent grade or better. Nucleotides, nucleotide analogs, ruthenium red, and Percoll were obtained from Sigma and used as provided. In the case of nucleotides and nucleotide analogs, purity was confirmed by HPLC analysis. The Li^+ salts of succinate, phosphate, 4-(2-hydroxyethyl)-1-piperozineethanesulfonic acid (Hepes),[1] EGTA, and EDTA were prepared by neutralizing the free acid forms with LiOH. The mannitol and sucrose used in mitochondrial media were deionized by passage over Amberlite MB-3, and stored subsequently at 4 °C in polyethylene containers.

Preparation of Mitochondria and Mitoplasts. Rat liver mitochondria were prepared from male Sprague-Dawley rats which weighed approximately 250 g, using a standard procedure (Broekemeier et al., 1985). The final pellets were suspended at approximately 60 mg of protein/mL in medium containing Li^+, rather than Na^+, Hepes.[2] These preparations, which were obtained by differential centrifugation only, contain small amounts of nonmitochondrial subcellular structures (e.g., Broekemeier et al., 1991; Gordon et al., 1994). They are referred to below as standard mitochondria.

Highly purified mitochondria were obtained from the standard preparations by a Percoll density-gradient procedure (Gordon et al., 1994). The purified mitochondria and contaminants were obtained as bands located approximately 1 cm from the bottom and the top of the gradient, respectively. Mitochondria were recovered from the lower band, by diluting it to 30 mL with washing medium [230 mM mannitol, 70 mM sucrose, 3 mM Hepes(Na^+), pH 7.4], and centrifuging at 6800*g* for 10 min. Washing was repeated 3 times to remove residual Percoll, and the final pellet was suspended at ∼45 mg of protein/mL in the Hepes(Li^+) medium.

Mitoplasts were prepared from standard or Percoll gradient-purified mitochondria by the French press technique, as follows: Mitochondria were suspended at 28 mg of protein/mL in washing medium which contained twice the normal concentrations of mannitol, sucrose, and Hepes(Li^+). After standing on ice for 30 min, the suspensions were loaded into a chilled French press mini cell (SLM Instruments, Rochester, NY) and extruded, using a constant-pressure French press supplied by SLM. The sample pressure was maintained at 1800−2000 psi during extrusion, while the flow rate was manually adjusted to ∼50 drops/min. The resulting mitoplasts were diluted 2-fold with the hyperosmotic medium and sedimented by centrifugation at 5000*g* for 10 min. The resulting pellet was washed once with the 300 mOsM medium, and finally suspended in the latter medium at ∼45 mg of protein/mL. Of the mitochondrial adenylate kinase activity, 90−100% was released by this procedure, whereas 85−95% of the malate dehydrogenase activity was recovered with the mitoplasts. These preparations retain high values of respiratory control and capacities for energy-linked functions (Broekemeier et al., 1991; Gordon et al., 1994).

For some experiments, mitoplasts or mitochondria were treated with chymotrypsin before use. In those cases, suspensions containing 28 mg of protein/mL were incubated at 25 °C in the 300 mOsM Li^+ medium which contained chymotrypsin at 10 units/mL. Activity was stopped after 2 min by diluting the suspension with 10 volumes of ice-cold medium that contained 0.5 mM phenylmethanesulfonyl fluoride (PMSF). After standing briefly on ice, the suspensions were centrifuged at 5000*g* for 10 min. The pellets were washed once in the absence of PMSF and suspended at ∼45 mg of protein/mL.

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[1] Abbreviations: AMPPNP, adenosine 5′-(β,γ-imidotriphosphate); AP_5A, P_1,P_5-di(adenosine-5′) pentaphosphate; CATR, carboxyatractyloside; CCP, carbonyl cyanide *p*-chlorophenylhydrazine; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Oligo, oligomycin; PMSF, phenylmethanesulfonyl fluoride; RuRed, ruthenium red.

[2] Li^+, rather than Na^+ or K^+, salts were used to limit possible involvement of transporters other than the uniporter during this study.

Determination of Reverse Uniport Activity. Mitochondria or mitoplasts were incubated at room temperature (~22−24 °C), at 1.0 mg of protein/mL, in a medium containing 10 mM succinate(Li^+), rotenone (0.5 nmol/mg of protein), and 3 mM Hepes(Li^+), pH 7.4, plus sufficient mannitol/sucrose (3:1 mole ratio) to give a total osmotic pressure of 300 mOsM. When utilized, spermine, adenine nucleotides, and nucleotide analogs were present from the beginning of the incubations or were added shortly after the mitochondria. After an initial 1.5 min preincubation, $SrCl_2$ (50 nmol/mg of protein) was added, and 4 min was allowed for the accumulation of Sr^{2+}. This was followed at 6.5 and 7.5 min by the addition of 0.6 mM EGTA(Li^+) and 5 nmol/mg of protein of carbonyl cyanide *p*-chlorophenylhydrazine (CCP), respectively. When Mg^{2+} was present, 1.2 mM $MgCl_2$ was added after Sr^{2+} accumulation and before the addition of EGTA. In the figures obtained from these experiments, time zero refers to the time of CCP addition, rather than the beginning of the incubations. Following addition of the uncoupler, 1.0 mL samples were taken at appropriate times for the determination of Sr^{2+} release. The mitochondria or mitoplasts were rapidly sedimented in a microcentrifuge, and Sr^{2+} released was determined by atomic absorption measurements carried out on the resulting supernatants. Values of Sr^{2+} release are expressed units of nanomoles per milligram of protein. The extramitochondrial Sr^{2+} level at the time of uncoupler addition varied from ~1 to 4 nmol/mg of protein over the range of conditions employed.

Other Methods. Protein was determined by the Biuret method using bovine serum albumin as a standard. The samples were solublized with deoxycholate, which was present at a final concentration of 1% by weight. Inorganic phosphate was determined by the method of Sanui (1974). When the phosphate content of mitochondria was to be determined, the samples were first sedimented because mannitol interferes with the assay (Ho & Pande, 1974).

RESULTS

Use of Reverse Activity Measurements To Investigate Regulation of the Ca^{2+} Uniporter. Previous work has shown that the kinetics of uniport-dependent divalent cation release from mitochondria (reverse uniport) reveal functional and regulatory features of this Ca^{2+} transport activity which are difficult to isolate during study of the forward reaction (Riley & Pfeiffer, 1986b). Of interest to the present report, EGTA, in conjunction with free external Mg^{2+}, strongly inhibits the release of Ca^{2+} or Sr^{2+} from uncoupled mitochondria, indicating that the uniporter is regulated through a divalent cation binding site which is accessible at the outer surface of the inner membrane. (Igbavboa & Pfeiffer, 1988, 1991a,b). However, uncoupling Ca^{2+}-loaded mitochondria while the uniporter is inhibited causes opening of the permeability transition pore (Igbavboa & Pfeiffer, 1988; Bernardi, 1992). This high-activity release pathway obscures any residual Ca^{2+} release which may be occurring *via* the uniporter (Riley, & Pfeiffer, 1985). As a result, Ca^{2+}-loaded mitochondria cannot be employed during the investigation of reverse uniport activity under many conditions of interest.

In contrast, when Sr^{2+}-loaded mitochondria are uncoupled while the uniporter is inhibited, the permeability transition pore remains closed as indicated by the absence of swelling or Mg^{2+} release, the failure of radiolabeled sucrose to enter the matrix space, and the absence of a cyclosporin A effect on residual rates of reverse Sr^{2+} uniport (Igbavboa & Pfeiffer, 1991a,b). Accordingly, Sr^{2+}-loaded, rather than Ca^{2+}-loaded, mitochondria were employed during this investigation.

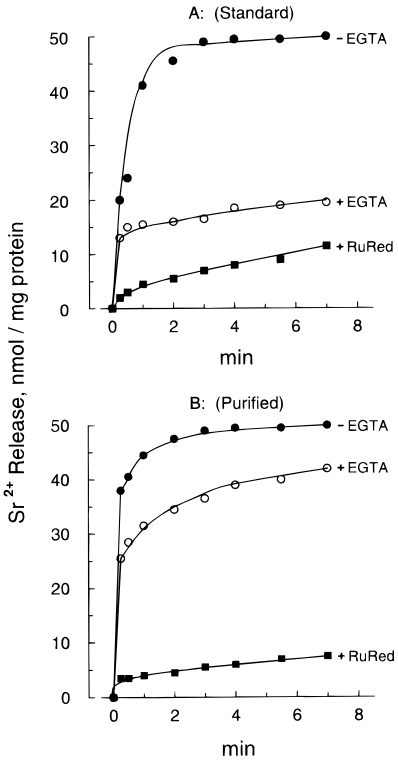

FIGURE 1: Mitochondrial purity influences the inhibition of reverse uniport activity by EGTA plus Mg^{2+}. Mitochondria were incubated at 1.0 mg of protein/mL in the succinate-containing medium described under Experimental Procedures. $SrCl_2$ (50 nmol/mg of protein) was added 1.5 min after the addition of mitochondria, followed at 5 min by $MgCl_2$ (1.2 mM). When utilized, EGTA (0.6 mM) or ruthenium red (2 nmol/mg of protein) was added at 6.5 min. At 7.5 min (designated 0 min in the figure), CCP (5 nmol/mg of protein) was added to initiate reverse uniport. Samples were taken at the times indicated for the determination of Sr^{2+} release as described under Experimental Procedures. Panel A was obtained using standard mitochondria (i.e., prepared by differential centrifugation alone). Panel B was obtained using gradient-purified mitochondria prepared as described under Experimental Procedures. For both panels: (●), neither ruthenium red nor EGTA was added; (○), EGTA was added; (■), ruthenium red was added.

Figure 1A illustrates the regulation of reverse uniport activity through the external divalent cation site under conditions relevant to the present study. The control experiment shows that when Sr^{2+}-loaded mitochondria are uncoupled in a medium containing 1.2 mM Mg^{2+}, Sr^{2+} is released rapidly, with >40 nmol/mg of protein being released during the first minute. Release occurs predominantly by reverse activity of the Sr^{2+} (Ca^{2+}) uniporter as shown by the inhibitory action of ruthenium red (Figure 1A). External EGTA is nearly as effective as ruthenium red, indicating that reverse uniport activity is strongly dependent upon the concentration of free Sr^{2+} (and/or Ca^{2+} from the endogenous content) in the external medium. In addition, when EGTA is employed, the kinetics of reverse uniport are biphasic. During the first 30 s, an initial rapid phase releases ~15 nmol/mg of protein of Sr^{2+}. This is followed by a slow phase during which further release proceeds at ~1 nmol min^{-1} (mg of protein)$^{-1}$ (Figure 1A). As in the control experiment (EGTA absent), the initial rapid phase seen in the presence of EGTA is subject to inhibition by ruthenium red, indicating

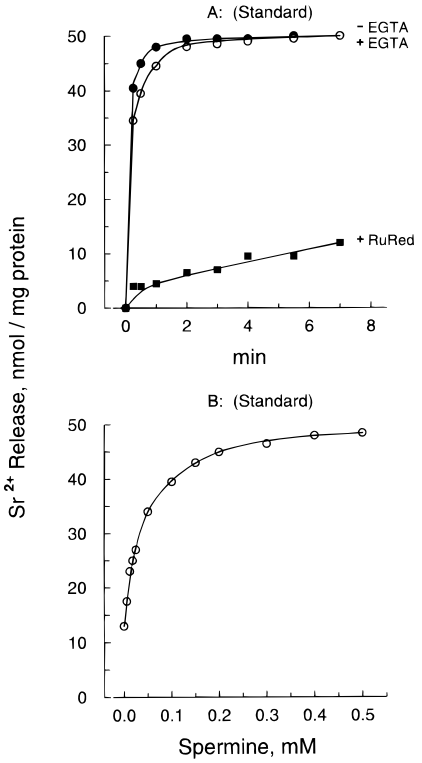

FIGURE 2: Effect of spermine on reverse uniport activity. Panel A: experiments were conducted using standard mitochondria as described under Experimental Procedures and the legend to Figure 1, except that 0.5 mM spermine was present from the beginning of the incubations. (●) Neither EGTA nor ruthenium red was added before the addition of uncoupler. (○) EGTA was added before uncoupler. (■) Ruthenium red was added before uncoupler. Panel B: data were obtained from experiments like those shown in panel A, EGTA added, except that the medium concentration of spermine was varied as shown. The values of Sr^{2+} release refer to the samples taken at 1 min following the addition of CCP.

that it also represents the activity of the uniporter operating in reverse (data not shown). The slower phase is again ruthenium red insensitive, indicating the involvement of other transporters (data not shown). Thus, following an initial period of ~30 s, the rate of uncoupler-induced reverse uniport is near zero in media containing excess EGTA and Mg^{2+}.

In the earlier reports, we established that inhibition of reverse uniport by EGTA is a function of the external Mg^{2+} concentration with ~0.5 mM required to produce a maximal effect (Igbavboa & Pfeiffer, 1988, 1991b). We also reported that EGTA is less effective when mitoplasts are employed instead of mitochondria, and that adding back the intermembrane space fraction obtained during mitoplast preparation restores the inhibition produced by Mg^{2+} plus EGTA (Igbavboa & Pfeiffer, 1991b). Additional factors which influence the regulation of reverse uniport by external divalent cations are illustrated in Figure 1B and Figure 2. EGTA is less effective as an inhibitor of reverse uniport when the contaminants in mitochondrial preparations have been reduced by use of Percoll density-gradient centrifugation (Figure 1B). The effect of mitochondrial purification is highly reproducible as can be illustrated by taking the amount of Sr^{2+} released during the first minute as an index. With standard mitochondria, the value of this index was 13 ± 3 nmol/mg of protein ($n = 21$), whereas with purified mitochondria the value was 32 ± 2 nmol/mg of protein ($n = 29$). Thus, mitochondria prepared by differential centrifugation alone contain an unidentified component which is required to obtain full inhibition of reverse uniport upon chelating external Sr^{2+} (and/or Ca^{2+}) in Mg^{2+}-containing media, and which is subject to reduction or removal during mitochondrial purification by the gradient procedure. In addition, when this component is absent or reduced, the kinetics of reverse Sr^{2+} uniport in the presence of external EGTA are pseudo-hyperbolic, as opposed to the biphasic behavior seen with standard mitochondria (compare Figure 1A and Figure 1B).

Spermine is known to activate or inhibit the uniporter when it is operating in the forward direction, depending upon conditions (Gunter & Pfeiffer, 1990; Rottenberg & Rustenbeck, 1990; Lenzen et al., 1992; Rustenbeck et al., 1993). Figure 2A shows that spermine also influences regulation of the reverse reaction by external divalent cations, in that the presence of 0.5 mM spermine eliminates the inhibition produced by external EGTA and Mg^{2+} (Figure 2A). The effect of spermine is concentration-dependent with a half-maximal effect obtained near 40 μM (Figure 2B).[3] As in the case of gradient-purified mitochondria, the kinetics of Sr^{2+} release are pseudo-hyperbolic when spermine is present. This is shown by the data in Figure 2A, which were obtained at 0.5 mM spermine, with EGTA and Mg^{2+} present. From these data and our earlier reports (Riley & Pfeiffer, 1986a,b; Igbavboa & Pfeiffer, 1988, 1991a,b), it is apparent that the uniporter is subject to complex regulation by external effectors, and that this regulation can be investigated under conditions of reverse activity.

Inhibition of Reverse Uniport by Adenine Nucleotides. In media containing EGTA plus Mg^{2+}, reverse uniport of Sr^{2+} is further inhibited by the presence of ATP as shown in Figure 3. Figure 3A illustrates this in the case of gradient-purified mitochondria; whereas in Figure 3B, standard mitochondria, were employed and 0.5 mM spermine was present to activate the reverse reaction. In both cases, the presence of 1.5 mM ATP reduces Sr^{2+} release to a level which is comparable to that seen with standard mitochondria when spermine is absent (compare to Figure 1A).[4]

A priori, there are several sites, mechanisms, and compounds which might contribute to the inhibitory effect of ATP. Since when obtaining Figure 3 the nucleotide was incubated with mitochondria prior to adding uncoupler, and since uncoupling will provoke ATP hydrolysis via the F_1F_o ATPase during the period of Sr^{2+} release, metabolites derived from ATP, rather than ATP per se, could be involved. Accordingly, the actions of ATP metabolites on reverse uniport were examined. Figure 4 shows that among the adenine nucleotides the order of effectiveness is ATP > ADP > AMP, regardless of whether gradient-purified mitochondria (Figure 4A) or standard mitochondria in a spermine-containing medium (Figure 4B) are employed. Adenosine has no effect with either type of preparation when tested at levels of 2.5 mM or below (data not shown). These findings suggest that ATP dominates in altering reverse uniport activity under the condition of Figure 3. With both types

[3] The fractional inhibition of reverse uniport produced by EGTA plus Mg^{2+} in gradient-purified mitochondria is also relieved by spermine, with a similar concentration dependence (data not shown).

[4] ATP also inhibits reverse uniport in gradient-purified mitochondria when spermine is present at a level sufficient to relieve the fractional inhibition produced by EGTA plus Mg^{2+} (data not shown).

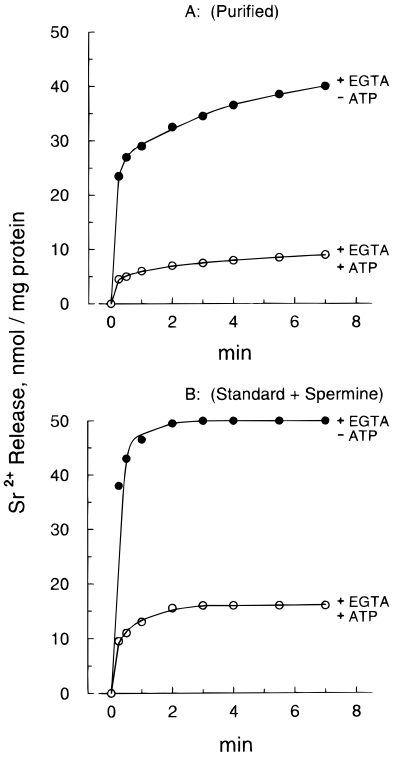

FIGURE 3: ATP inhibits reverse uniport activity. Experiments were conducted as described under Experimental Procedures and the legend to Figure 1. Panel A was obtained using gradient-purified mitochondria. Panel B was obtained using standard mitochondria with 0.5 mM spermine present to activate reverse uniport of Sr^{2+}. For both panels: (●) control conditions for which ATP was absent; (○) ATP was present at 1.5 mM and was added at 1 min following the addition of mitochondria.

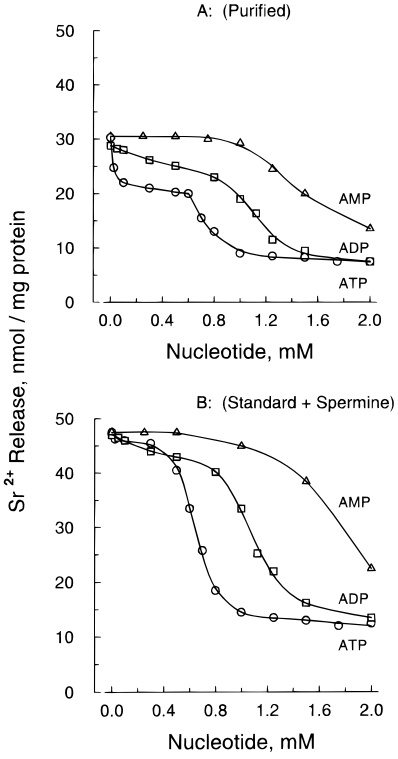

FIGURE 4: Concentration dependence of reverse uniport inhibition by adenine nucleotides. Data were obtained from experiments like those shown in Figure 3, except that the adenine nucleotide utilized and its concentration were varied. Panel A was obtained using gradient-purified mitochondria; whereas for panel B standard mitochondria were used and the medium contained 0.5 mM spermine. For both panels, the nucleotides utilized were as follows: (○) ATP; (□) ADP; (△), AMP. The Sr^{2+} release values were obtained from samples taken at 1 min after the addition of uncoupler.

of mitochondria, the EC_{50} for ATP is ~0.6 mM; however, the concentration dependencies are otherwise dissimilar. In the case of standard mitochondria incubated in the presence of spermine, the ATP inhibition curve displays a sigmoidal shape with little or no effect of ATP seen below a concentration of ~0.4 mM (Figure 4B). With gradient-purified mitochondria, the curve is complex, seemingly composed of two segments within which the dependency of inhibition on ATP concentration is hyperbolic. About 50% of the inhibitory effect is obtained within each segment, with the first and second segments approaching completion at 0.2 and 1.0 mM ATP, respectively (Figure 4A). This difference between standard and gradient-purified mitochondria is seen consistently under the conditions of Figure 4. In contrast to ATP, the inhibition curves obtained with ADP and AMP display similar shapes with both types of mitochondrial preparation (Figure 4).

The effect of medium phosphate concentration on reverse Sr^{2+} uniport was also investigated because under the conditions of Figure 3, the hydrolysis of adenine nucleotides would increase the phosphate concentration. Figure 5 shows that phosphate inhibits reverse uniport in gradient-purified mitochondria, but has no effect when standard mitochondria are used in a spermine-containing medium. When using purified mitochondria, the EC_{50} with respect to exogenous phosphate is ~20 μM, or 20 nmol/mg of protein under the conditions of Figure 5. This value can be compared to the endogenous phosphate contents of standard and gradient-purified mitochondria which were found to be 2−3 and ~1 nmol/mg of protein, respectively.

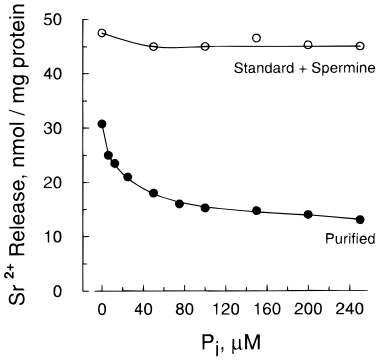

FIGURE 5: Effect of medium phosphate concentration on reverse uniport activity. Data were obtained from experiments like those shown in Figure 3 except that inorganic phosphate, rather than ATP, was present in the medium at the concentrations indicated. The Sr^{2+} release values were obtained from samples taken at 1 min after the addition of uncoupler. (●), Gradient-purified mitochondria were used. (○) Standard mitochondria were used, and the medium contained 0.5 mM spermine.

Actions of Inhibitors, Other Nucleotides, and Nucleotide Analogs. To further test the possibility that ATP metabolites contribute to the inhibition of reverse uniport by ATP, the studies summarized in Table 1 and Figure 6 were carried out. When present throughout the experimental period, oligomycin, AP_5A, and carboxyatractyloside, used alone or in combinations, have no effect on the inhibitory action of ATP. This is true regardless of whether gradient-purified mitochondria, standard mitochondria in the spermine-

Table 1: Effect of Selected Inhibitors on Reverse Uniport Activity in the Absence and Presence of ATP[a]

addition	preparation		
	gradient-purified mitochondria	standard mitochondria	mitoplasts
none			
−ATP	31.5 ± 1.0	48.7 ± 0.6	32.6 ± 0.9
+ATP	5.7 ± 0.2	14.3 ± 0.9	5.7 ± 0.3
AP_5A			
−ATP	32.7 ± 1.3	48.2 ± 1.4	31.6 ± 0.6
+ATP	6.9 ± 0.4	13.5 ± 0.6	5.6 ± 0.3
Oligo			
−ATP	32.9 ± 0.7	48.8 ± 1.1	32.1 ± 0.8
+ATP	7.0 ± 1.0	14.7 ± 0.8	6.6 ± 0.3
CATR			
−ATP	32.5 ± 1.1	48.5 ± 1.2	32.5 ± 0.5
+ATP	7.3 ± 0.3	14.5 ± 0.6	7.1 ± 0.7
Oligo + CATR			
−ATP	33.2 ± 0.3	48.9 ± 0.7	33.5 ± 0.8
+ATP	7.4 ± 0.7	14.9 ± 0.4	7.1 ± 0.3

[a] Gradient-purified mitochondria and mitoplast data were obtained in the absence of spermine, whereas 0.5 mM spermine was present in the case of standard mitochondria. The Sr^{2+} release values shown are in units of nmol/mg of protein and were obtained as described in the legend to Figure 3, using samples taken at 1 min after the addition of uncoupler. Each value is the mean of four determinations, ±SD, with the individual values obtained using separate preparations. When present, 1.5 mM ATP was added 1 min following the addition of mitochondria. Inhibitors were added at the beginning of the incubations, 30 s prior to the ATP addition. The inhibitor levels were as follows: AP_5A, 50 μM; oligomycin, 2 μg/mg of protein; carboxyatractyloside, 1 nmol/mg of protein.

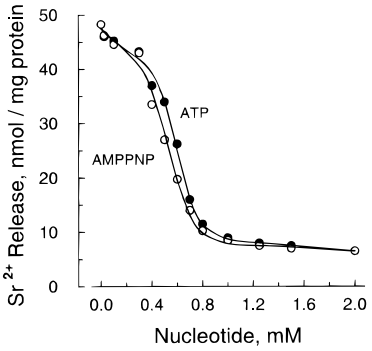

FIGURE 6: Comparison of ATP and AMPPNP as inhibitors of reverse uniport activity. Data were obtained using standard mitochondria in the presence of 0.5 mM spermine, as described in the legend to Figure 4, except that oligomycin (2 μg/mg protein) and carboxyatractyloside (1 nmol/mg protein) were present. ATP (●) or AMPPNP (○) was present throughout the incubations at the indicated concentrations.

containing medium, or mitoplasts prepared from gradient-purified mitochondria are employed (Table 1). In the case of standard mitochondria incubated in the presence of spermine, the failure of oligomycin and/or carboxyatractyloside to relieve the inhibition produced by ATP confirms Figure 4 in indicating that hydrolysis of ATP is not required to obtain the inhibitory effect. This finding is also confirmed by Figure 6 which shows that the analog AMPPNP is as effective as ATP when investigated side by side using standard mitochondria in the spermine-containing medium. As regards gradient-purified mitochondria, the failure of oligomycin and/or carboxyatractyloside to influence the inhibition produced by ATP shows that the action of phosphate seen in Figure 5 is not primarily responsible for the effect of ATP under the conditions of Figure 3. AMPPNP could not be tested on gradient-purified mitochondria because it contained contaminating phosphate at a mole ratio of ~1:50 with respect to the analog (data not shown). Thus, as the AMPPNP concentration was increased in incubations of gradient-purified mitochondria, the increase in medium phosphate concentration alone was sufficient to diminish the reverse uniport of Sr^{2+} (see Figure 5).

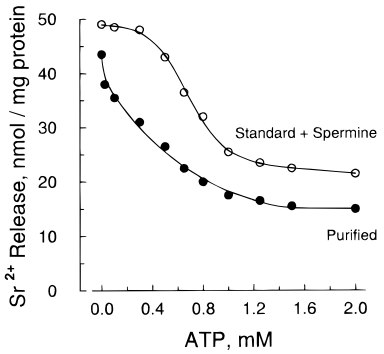

FIGURE 7: Inhibition of reverse uniport activity by ATP in the absence of free Mg^{2+}. Data were obtained from experiments like those shown in Figure 4 except that $MgCl_2$ was not added, EDTA was used instead of EGTA, and oligomycin (2 μg/mg protein) was present. The ATP concentration was varied as shown, and samples were taken at 1 min after the addition of uncoupler. (●) Gradient-purified mitochondria were used. (○) Standard mitochondria were used, and the medium contained 0.5 mM spermine.

The actions of nucleotide triphosphates other than ATP were also examined using gadient-purified mitochondria incubated in the presence of spermine. Experiments like those shown in Figure 4 demonstrated that the order of effectiveness is ATP > CTP ≈ UTP > GTP.

Interaction between ATP and Mg^{2+} as Inhibitors of Reverse Uniport. Apart from the points noted above, the data in Table 1 indicate that the outer mitochondrial membrane, the intermembrane space adenylate kinase activity, and the inner membrane adenine nucleotide translocase activity are not required to obtain inhibition of reverse Sr^{2+} uniport by ATP. Thus, ATP may act at the uniporter *per se*, by association at a site which is accessible directly from the intermembrane space. The site at which Mg^{2+} acts to inhibit reverse uniport is also located on the outer side of the inner membrane (Igbavboa & Pfeiffer, 1988, 1991a,b). Since ATP will be fractionally complexed to Mg^{2+} when both are present, there are several possible relationships between the sites at which Mg^{2+} and ATP influence the uniporter. Regarding these, Figure 7 shows that ATP inhibits reverse uniport even when Mg^{2+} is not added to the medium and when EDTA is used instead of EGTA to chelate external Sr^{2+} (Mg^{2+}). This is true both with gradient-purified mitochondria and with standard mitochondria incubated in the spermine-containing medium, demonstrating that free ATP is an effective inhibitor. However, a comparison of Figures 7 and 4 shows that free ATP is less effective than ATP and Mg^{2+} used in combination. In addition, the ATP inhibition curve obtained with gradient-purified mitochondria (Figure 7) does not show the biphasic feature which is seen when Mg^{2+} is present (Figure 4). Thus, the Mg−ATP complex may also be an effective inhibitor.

Further insight into regulatory relationships between Mg^{2+} and ATP arises from Figure 8 which shows that treatment of mitoplasts with chymotrypsin markedly diminishes the inhibitory effect of free Mg^{2+}, but has no effect on the inhibition produced by free ATP. As will be further

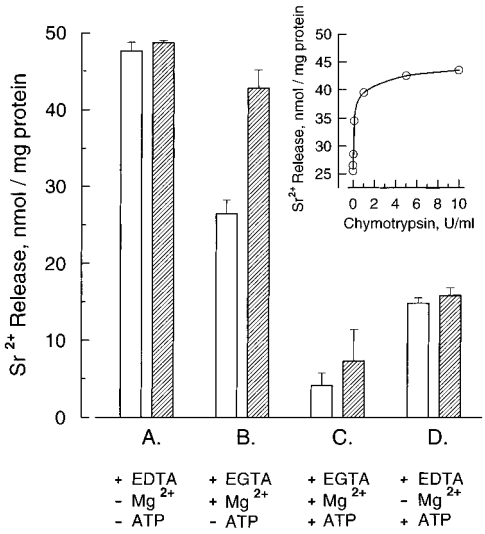

FIGURE 8: Chymotrypsin treatment diminishes Mg^{2+}-dependent inhibition of reverse uniport activity. Mitoplasts prepared from standard mitochondria were treated with chymotrypsin at 10 units/mL as described under Experimental Procedures. They were then used to determine the effects of Mg^{2+} and ATP on reverse uniport activity as described in the legends to other figures. For group A, $MgCl_2$ and ATP were not added, and EDTA was used instead of EGTA. For group B, EGTA was used, and $MgCl_2$ was added at 1.2 mM, but ATP was not added. For C, conditions were the same as for B, except that 1.5 mM ATP was also present. For D, conditions were the same as for A, except that 1.5 mM ATP was present. Oligomycin (2 μg/mg of protein) was present in all cases. In each group, the open and hatched bars were obtained with control and chymotrypsin-treated mitoplasts, respectively. The Sr^{2+} release values were obtained from samples taken at 1 min after the addition of uncoupler. Each value is the mean of three determinations, with the individual values obtained with separate preparations of mitoplasts. The standard deviations of the mean values are shown for each condition. For group B, statistical analysis (Student's *t*-test) showed that the control and treated values are significantly different ($p < 0.001$). For all other groups, the differences are not significant. The insert panel was obtained under the conditions described for group B, using chymotrypsin-treated mitoplasts prepared by the normal procedure, except that the chymotrypsin activity was varied as shown.

described below, this finding indicates that Mg^{2+} and ATP act at different sites, although the ATP site may also accept the Mg−ATP complex. In contrast to mitoplasts, chymotrypsin treatment of mitochondria had no effect on the inhibitory action of Mg^{2+} or of ATP, confirming that the protease-sensitive site is associated with the inner membrane (data not shown).

DISCUSSION

A Model for Regulation of the Uniporter by Divalent Cations and Nucleotides. For interpretation of the present data, we adopt the view that the mitochondrial Ca^{2+} uniporter transports by a gated channel type of mechanism. The evidence supporting this has been reviewed (Gunter & Pfeiffer, 1990). It is based upon an apparent turnover number of ~20 000/s (too fast for a carrier mechanism, too slow for a channel which is not gated), a dependence of Ca^{2+} influx through the uniporter on membrane potential (i.e., a voltage dependence), and the absence of saturation transport kinetics as a function of Ca^{2+} concentration, within the bounds imposed by the maximal activity of the mitochondrial electron transport chain.

Within the context of a gated-channel mechanism, inhibition of reverse uniport in uncoupled mitochondria reflects the closure of channels which were open prior to uncoupling, before all Sr^{2+} has been released. The amount of Sr^{2+} released before the closure of all channels reflects the number which were open at the time reverse activity was initiated, the time required to close them, and the rate of Sr^{2+} flux through these channels during the intervening period. The manual sampling methods applied here cannot distinguish which of these parameters is altered by a particular effector. Single-channel methods, such as the patch clamp technique, presumably could distinguish. However, investigators utilizing these methods have not reported an ion-conducting channel in the inner mitochondrial membrane which displays the characteristics expected of the Ca^{2+} uniporter. This may reflect the existence of Ca^{2+}-regulated permeability transition pores which are found in nearly all patches (Zoratti & Szabó, 1995). These pores have a very large conductance for Ca^{2+} and other ions (Zoratti & Szabó, 1995), which would obscure any conductance arising from the uniporter. Since single-channel methods are not applicable at present, the terms "rapid closure" and "slow closure" are used below with the understanding that they reflect summed effects of several activity parameters, expressed within a large population of uniporter channels, that are seen as an ensemble average.

The model shown in Figure 9 depicts the uniporter as a gated channel that is controlled by effector binding sites. Site 1 is a cation binding site which accepts Sr^{2+}, Ca^{2+}, Mg^{2+}, and perhaps other divalent cations. When the site is occupied by Sr^{2+} (Ca^{2+}) and a membrane potential is present (i.e., when the mitochondria are energized), the channel is open and allows cations to move down their electrochemical gradient, in accordance with a selectivity sequence established by site 3 (uppermost form in Figure 9). Collapse of the membrane potential upon uncoupling changes the open/closed probability in a fashion which is determined, in part, by the status of site 1. When the site is not occupied (e.g., when EDTA is present), or when it remains occupied by Sr^{2+} (Ca^{2+}) (e.g., when no chelator is present), the channel remains open, and all previously accumulated Sr^{2+} (Ca^{2+}) is promptly released as in Figure 1A, EGTA absent. This form of the uniporter is labeled A in Figure 9. When site 1 is occupied by Mg^{2+} following uncoupling (e.g., when EGTA plus external Mg^{2+} are present), the channel closes rapidly, such that a fraction of previously accumulated Sr^{2+} (Ca^{2+}) is released within seconds but subsequent release is very slow, as in Figure 1A, EGTA present. Form B in Figure 9 illustrates this form of the uniporter.

Regulation of the uniporter through site 1, as depicted in Figure 9, is fully consistent with our previous reports on the effects of external divalent cation conditions on reverse uniport activity (Igbavboa & Pfeiffer, 1988, 1991a,b). In those reports, divalent cations were also proposed to act at an external regulatory site. In the present model, the channel characteristic of the uniporter is recognized, and the regulatory cations are allowed to influence the rate of inactivation, as opposed to simply favoring an active or inactive form. The rate parameter is introduced because under some conditions reverse uniport is fast initially, and then abruptly slows down or stops (e.g., Figures 1 and 3; Igbavboa & Pfeiffer, 1991a,b). In addition, the fraction of cations released during the rapid phase can vary with conditions. Behavior of this type is allowed if the regulatory cations change the

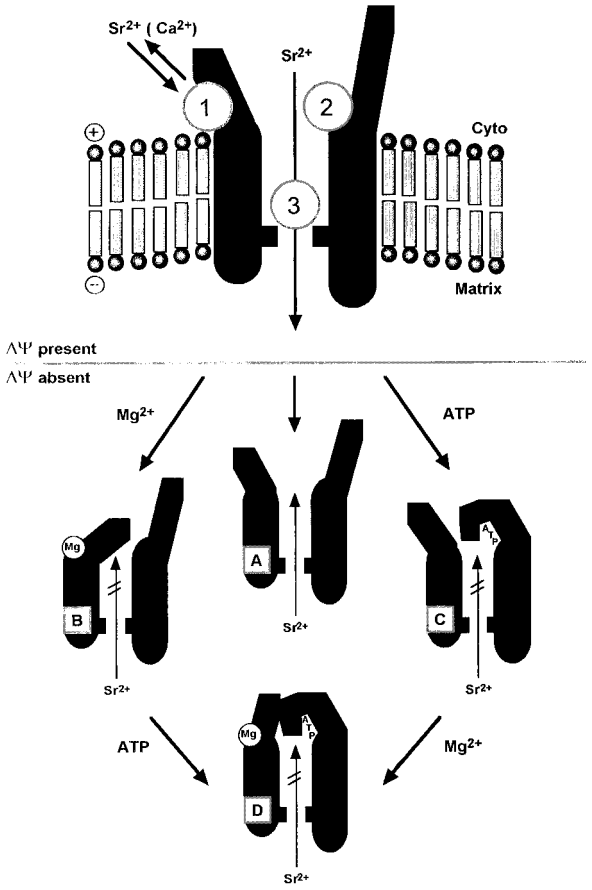

FIGURE 9: Model of uniporter regulation by nucleotides and divalent cations. The binding sites labeled 1 and 2 accept divalent cations and nucleotides (or cation-nucleotide complexes), respectively. The site labeled 3 determines the channel selectivity. The upper part of the figure illustrates an open uniporter in a mitochondrion which is energized. Collapse of the membrane potential upon uncoupling leads to various forms of the uniporter depending upon the status of sites 1 and 2, as illustrated in the lower portion of the figure and further described under Discussion. Form A allows Sr^{2+} efflux from the mitochondria. B−D are closed forms and do not allow efflux, although some efflux may occur initially during the time interval between uncoupling and generation of the closed forms.

rate of channel closure. If this parameter is not influenced, it must be assumed that the cations bind and debind to site 1 very slowly, which is not expected for Ca^{2+}, Sr^{2+}, or Mg^{2+}, from a chemical perspective.

The present data shows that external adenine nucleotides, such as ATP, also regulate the uniporter. Within the context of the gated-channel model, external nucleotides behave like Mg^{2+} and cause the uniporter to close following uncoupling, before all previously accumulated Sr^{2+} is released (Figure 3). In Figure 9, ATP is shown as acting at a distinct site (site 2) to produce the closed form which is labeled C. A final form labeled D is also shown in which both sites 1 and 2 are occupied by their respective ligands. The evidence that free Mg^{2+} and free ATP act at distinct sites includes the fact that either one is effective in the absence of the other (Figures 1 and 7) while they are chemically dissimilar and so unlikely to associate with a given site individually, although association as the Mg−ATP complex would be possible. The strongest evidence for distinct sites, however, is loss of the free Mg^{2+} action upon treatment of mitoplasts with chymotrypsin, without an effect on the action of free ATP (Figure 8).

Affinities and the Form of Regulation. From previous studies, the EC_{50} value for Mg^{2+} as an inhibitor of reverse uniport in the absence of ATP is ∼50 μM (Igbavboa & Pfeiffer, 1991b). Within the model shown in Figure 9, this value represents the affinity of site 1 for Mg^{2+} at pH 7.4. The affinity of this site for Ca^{2+} or Sr^{2+} is clearly much higher because the external free Sr^{2+} (Ca^{2+}) concentrations arrived at as the extent of accumulation becomes maximal (∼μM) are sufficient to maintain an open channel upon uncoupling, even when the external Mg^{2+} concentration is 1.2 mM (Figure 1A). An exact value cannot be determined from existing data; however, it should be possible to determine it by varying the external Sr^{2+} (Ca^{2+})/Mg^{2+} ratio at the time of uncoupling, and comparing the kinetics of subsequent reverse uniport to these ratios.

When standard mitochondria in the spermine-containing medium are considered, the EC_{50} value for ATP acting at site 2 is ∼0.6 mM as can be seen in Figures 4B, 6, and 7. ATP is apparently acting through an allosteric type of mechanism, as opposed to a mechanism involving a protein kinase. This is indicated by the fact that ATP and AMPPNP are equally effective (Figure 6), together with the effectiveness of ATP in the absence of free Mg^{2+} (Figure 7). However, the involvement of kinases cannot be ruled out completely until the biphasic inhibition curve seen with gradient-purified mitochondria (Figure 4A) can be explained (see further discussion below). If it is assumed that kinases are not involved, the biphasic characteristic together with the sigmoidal shape of the ATP inhibition curve seen with standard mitochondria when spermine is present (Figures 4B, 6, and 7) allows the possible existence of two adenine nucleotide sites which are cooperative, or differently able to accept the free and Mg^{2+}-complexed forms of ATP.

Actions of Other Effectors. Several aspects of the present data show that factors other than cations and nucleotides acting through sites 1 and 2 are important as regulators of the uniporter. These include the effects of spermine and phosphate and the multiple effects of mitochondrial purity. Within the context of the model shown in Figure 9, spermine favors an open uniporter such that following uncoupling the fraction of Sr^{2+} released before the closure of all channels increases as the spermine concentration increases (Figure 2). Spermine may antagonize or slow the formation of closed form B (Figure 9), because the Mg^{2+}-dependent inhibition of reverse uniport is lost in the presence of sufficient spermine (Figure 2), while ATP remains active (Figures 3, 4, 6, and 7). Within the context of the model, the latter observation means that closed form C can arise rapidly, even when spermine is present.

The actions of phosphate and the apparent actions of mitochondrial contaminants seem more complicated than those of spermine. Phosphate can be seen as accelerating channel closure upon uncoupling purified mitochondria, but not standard mitochondria used in the spermine-containing medium (Figure 5). The uniporter may then contain a regulatory site which accepts phosphate, and which is further influenced by the contaminants or spermine. Considering the concentration dependence of the phosphate effect (Figure 5), it is also possible that a lower matrix pH arising from phosphate/hydroxide exchange is involved in producing the action of phosphate. However, it is not clear why gradient purification would change a matrix pH dependence. Another factor to consider is the expected effect of phosphate on the

matrix-free Sr^{2+} (Ca^{2+}) concentration at the time of uncoupling. This value is probably lowered when phosphate is present and accumulated with Sr^{2+}. Initial flux through the uniporter following uncoupling, before a closed form is attained, could then be decreased progressively as the phosphate concentration increases, producing an inhibition curve like the one shown in Figure 5. By this explanation, the absence of phosphate inhibition when spermine is present would reflect the slow channel closure caused by spermine, allowing sufficient time for complete release, even when the rate of flux through individual channels is low.

Regarding the action of contaminants, within the context of the model, closed form B is attained more rapidly upon uncoupling when contaminants are present (Figure 1). We are currently trying to identify the active component. Attempts to recover the behavior of standard mitochondria by adding contaminant fractions to gradient-purified mitochondria have so far produced variable results, wherein activity is dependent upon storage conditions. Although the effects of removing mitochondrial contaminants cannot yet be explained, they are reported here because they bear upon the interpretation of earlier data which suggested that a soluble intermembrane space component is required to obtain inhibition of reverse uniport in the presence of external EGTA and free Mg^{2+} (Igbavboa & Pfeiffer, 1991b). In that study, mitoplasts were prepared from standard mitochondria and would have been less contaminated than the mitochondria from which they were prepared [i.e., structures adhering to the outer membrane are largely removed by the mitoplast preparation procedure (Broekemeier et al., 1991)]. No loss of reverse uniport inhibition is seen upon removal of the outer membrane when gradient-purified mitochondria, and mitoplasts prepared from them, are compared (Table 1). In addition, protease treatment of mitoplasts can reduce or eliminate the Mg^{2+}-dependent inhibition of reverse uniport in uncoupled mitoplasts (Figure 8). Thus, lowered levels of contaminants and the action of proteases which they contain, rather than loss of an intermembrane space factor, probably account for the previous observation.

Implications. The regulatory features of mitochondrial Ca^{2+} transport activities must be fully elucidated if we are to understand the interplay between mitochondria and other cell components in cell signaling systems involving Ca^{2+}, and the role of Ca^{2+} in controlling ATP production (see the introduction). Literature on the regulatory features of the Ca^{2+} uniporter is somewhat phenomenological, and it is hoped that the specific model proposed here will lead to more detailed descriptions. The recently identified rapid Ca^{2+} accumulation mode (RAM) which is seen in isolated mitochondria subjected to Ca^{2+} pulses (Sparagna et al., 1995) is a particular activity which might be attributable to channel and gating characteristics of the uniporter. The same is true for the slow accumulation modes which are seen when mitochondria are deenergized and incubated in the absence of free Ca^{2+} prior to initiating Ca^{2+} accumulation (Kasparinsky & Vinogradov, 1996).

Regulation of the Ca^{2+} uniporter by adenine nucleotides suggests that the phosphorylation potential of the cell influences the extent to which the TCA cycle is accelerated by transference of excitatory Ca^{2+} pulses from the cytoplasm to the mitochondrial matrix space. Two scenarios can be envisioned. In the first, a low phosphorylation potential would render the uniporter more active, causing more Ca^{2+} to be accumulated in response to a pulse having a given set of characteristics. The activation would arise because ADP and AMP are less effective than ATP at generating closed form C (Figure 4). This interpretation is logical since a low ATP level reflects the need to accelerate TCA cycle activity to a higher rate than is required when the phosphorylation potential is high. The second scenario takes account of the phosphate and Mg^{2+} effects on uniporter activity, as well as those of nucleotides. Lowering the phosphorylation potential increases the phosphate and free Mg^{2+} levels, while decreasing the ATP/ADP ratio. Since rising phosphate and Mg^{2+} levels and falling ATP/ADP ratios have opposing effects on uniporter activity, the net effect might be to maintain a nearly constant activity. This interpretation allows the relationship between uniporter activity and cytoplasmic Ca^{2+} concentration to remain constant as the phosphorylation potential varies, as may be required. Further investigation of the model shown in Figure 9 should help to distinguish between these interpretations, which could apply alternatively, depending upon the cell type and the condition considered.

ACKNOWLEDGMENT

We thank Ronald Louters and Clifford Chapman for expert technical assistance with portions of this work.

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BI970180Y