On the interactions of Ca^{2+} and cyclosporin A with a mitochondrial inner membrane pore: a study using cobaltammine complex inhibitors of the Ca^{2+} uniporter

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The mitochondrial inner membrane contains a Ca^{2+}-activated pore of possible relevance to the pathogenesis of ischaemia/reperfusion injury which is inhibited by the immunosuppressant cyclosporin A (CSA). The present study employs a number of novel cobaltammine complex inhibitors of the Ca^{2+} uniporter (mediating Ca^{2+} uptake) to examine whether intramitochondrial Ca^{2+} influences the capacity of CSA to block the pore. Using dissipation of the inner membrane potential as a means of monitoring the state of the pore, it is shown that CSA blockade is facilitated as Ca^{2+} uptake is restricted. Ca^{2+} also depresses and reverses the binding of [^{3}H]CSA to mitochondria, but Ca^{2+} is ineffective when its uptake is prevented. It is concluded that a high intramitochondrial Ca^{2+} concentration antagonizes pore inhibition by CSA. The significance of this is discussed.

INTRODUCTION

The mitochondrial inner membrane contains a structure that forms a non-selective pore of about 20 Å (2 nm) diameter when triggered by high matrix concentrations of Ca^{2+} and P_i, and by oxidative stress [1–4]. Pore opening is fully reversible, and rapid pore closure occurs on Ca^{2+} removal [5]. Pore opening is blocked by physiological concentrations of ATP (> 1 mM [6]). The pore has been detected not only in isolated mitochondria but also as a very-high-conductance channel in patch-clamped mitoplasts [7]; these studies have revealed that the pore flickers between the open and closed states, possibly via one or more substate(s) [8]. The molecular identity of the pore and its native function have not been resolved. There is evidence that the open pore may involve a deformed adenine nucleotide translocase [3,4], and an association of this protein with porin of the outer membrane has been suggested [9]. High cellular Ca^{2+}, low ATP, high P_i and oxidative stress are features of ischaemia/reperfusion injury and, in our view, pore opening may be a factor in the pathogenesis of this form of injury [2,10]. In particular, pore opening uncouples mitochondrial energy transduction and would be expected to compromise cell viability severely.

The immunosuppressant cyclosporin A (CSA) blocks the pore in intact mitochondria [4,10] and in excised patches of the inner membrane [7]. CSA also markedly retards the onset of reoxygenation/reperfusion-induced injury in heart cells [11] and in the perfused heart [12]. The molecular target of CSA has not been identified, although one possible candidate is the CSA binding protein cyclophilin [4,13,14]. In view of its potential use in identifying components of the pore and in probing the pathophysiological role of the pore in the cell, it is important to establish the factors that influence CSA binding. The present study reports novel cobaltammine inhibitors of the Ca^{2+} uniporter (mediating Ca^{2+} influx) and their use in investigating the influence of matrix Ca^{2+} on CSA binding. It is shown that CSA binding to the relevant component of the pore system is markedly antagonized by high intramitochondrial Ca^{2+} concentrations.

MATERIALS AND METHODS

Mitochondrial preparation and assay

Mitochondria were isolated from the livers of male Sprague–Dawley rats (200–250 g body weight) and their protein content was determined as described previously [2]. They were suspended finally in 210 mM mannitol, 70 mM sucrose and 10 mM Tris/HCl (pH 7.2).

The mitochondrial inner membrane potential ($\Delta\psi$) was estimated from the accumulation of the tetraphenylphosphonium ion (TPP^+) using a TPP^+-sensitive electrode as described [10]. The basic medium (pH 7.0) contained 120 mM KCl, 10 mM Hepes, 1 mg of mitochondrial protein/ml, 1 μg of rotenone/ml, 5 mM KH_2PO_4 and 1 μM TPP^+. Further additions are given in the legends to the Figures and Tables.

The rates of Ca^{2+} uptake by respiring mitochondria were determined as described by Goldstone et al. [15] in medium containing 120 mM KCl, 10 mM Hepes, 1 mg of mitochondrial protein/ml, 1 μg of rotenone/ml, 0.5 mM KH_2PO_4, 60 μM Arsenazo III, 6 mM succinate and 0.2 mM ATP; uptake was started by addition of 10 μM $CaCl_2$. Rates were determined by measuring the time taken to decrease external Ca^{2+} from 5 μM to 3 μM and were corrected for the Na^+-independent rate of Ca^{2+} efflux (< 1 nmol Ca^{2+}/min per mg of protein) as stated previously [15].

[^{3}H]CSA binding to mitochondria was measured according to McGuinness et al. [14] in medium comprising 120 mM KCl, 10 mM Hepes (pH 7.2), 1 mg of mitochondrial protein/ml, 1 μg of rotenone/ml, 0.5 mM KH_2PO_4, 6 mM succinate, and either 1 mM EGTA or 25 μM $CaCl_2$. Following incubation for 1 min, [^{3}H]CSA was added; binding was measured 8 min later. Modifications to this standard procedure are stated in the Table legends (see Table 2).

Abbreviations used: TPP^+, tetraphenylphosphonium ion; $\Delta\psi$, mitochondrial inner membrane potential; A, NH_3 (in ammine complexes); CSA, cyclosporin A.

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Syntheses of cobaltammine complexes

For simplicity, NH_3 is generally abbreviated to A in formulae of ammine complexes (except in Table 1).

$[CoA_6]Cl_3$ was purchased from Aldrich. $[Co(H_2O)A_5](ClO_4)_3$ was prepared as described by Isied et al. [16] and was used as starting material for the other ammine complexes. $[Co(Ala)A_5]Br_3$ was synthesized from 0.45 g of $[Co(H_2O)A_5](ClO_4)_3$ (1 mmol) and 0.26 g of β-alanine (2.8 mmol) by the protocol of Bagger et al. [17] for the introduction of pentamminecobalt as a carboxyl protective group. The red/pink solid was crystallized from methanol; yield, 0.17 g or 36%. Calculated for $[(C_3H_7O_2N]Co(NH_3)_5Br_3$: C, 7.64; H, 4.67; N, 17.83; Br, 50.70. Found: C, 7.78; H, 4.60; N, 17.21; Br, 49.33. [Co(1,5-pentanediamine)A_5]Br_4 was prepared by a modification of the procedure of Ogino [18] as follows: 9 g of $[Co(H_2O)A_5](ClO_4]_3$ (19.5 mmol) was dissolved in 100 ml of dimethyl sulphoxide and mixed with 1.9 g of $NH_2(CH_2)_5NH_2$ (18.6 mmol). The mixture was heated immediately at 80 °C for 25 min, then cooled, neutralized (conc. HCl), and diluted with 700 ml of water. The solution was fractionated on an SP-Sepharose C-25 column (18 cm × 2.8 cm) eluted with a gradient of NaBr. The orange fraction eluting at 0.9–1.1 M NaBr (500 ml) was collected and then substantially freed of NaBr by binding to a Chelex column and elution with a small volume of 0.1 M HBr. The solution was neutralized (NaOH), and then left at 4 °C. The orange crystals were collected and dried over H_2SO_4; yield 1.64 g or 14.6%. Calculated for $[(C_5H_{15}N_2)Co(NH_3)_5]Br_4$: C, 10.59; H, 5.29; N, 17.29; Br, 56.36. Found: C, 10.19; H, 5.18; N, 16.98; Br, 55.14.

RESULTS

Pore opening and its blockade by CSA and Ruthenium Red

Ca^{2+}-induced opening of the inner membrane pore is associated with a non-selective increase in permeability to low-M_r solutes (e.g. sucrose [2]), collapse of the $\Delta\psi$ [10] and changes in light scattering [4]. In the present study, pore opening was monitored from the collapse of $\Delta\psi$, which was adequate to establish requirements for pore closure (see below). The procedure is illustrated in Figure 1(a). On commencement of respiration (using succinate), a $\Delta\psi$ of about 160 mV was developed; thereafter $\Delta\psi$ was dissipated on addition of excess Ca^{2+} (pore opening) and then largely restored on Ca^{2+} chelation with EGTA (pore closure). It is relevant to stress that direct measurements of pore state based on the entry and entrapment of [^{14}C]sucrose in the matrix space have confirmed that the reversible collapse of $\Delta\psi$ under these conditions is due to the opening and closure of the Ca^{2+}-dependent pore [19]. Numerous studies have established that pore activation by Ca^{2+} is blocked by the immunosuppressant CSA [4,7,10,14]. For the sake of clarity, this is also shown in Figure 1(a) (broken line). In the presence of CSA, Ca^{2+} caused only a transient decrease in $\Delta\psi$, reflecting the (transient) net uptake of Ca^{2+} across the inner membrane [19]. However, once pore opening had occurred, the subsequent addition of CSA was quite ineffective and $\Delta\psi$ remained dissipated (Figure 1a). In other words, although CSA prevented pore opening, it was unable to reverse pore opening once it had occurred, at least to the extent of allowing the restoration of $\Delta\psi$.

The loss of effectiveness of CSA as a pore inhibitor has been observed before and attributed to the depletion of intramitochondrial adenine nucleotides on pore opening [20]; as discussed previously, it seems that matrix nucleotides, in particular ADP, very markedly facilitate pore blockade by CSA. Here we report the novel observation that the capacity of CSA to block the pore is also restored by Ruthenium Red. Thus, whereas CSA and Ruthenium Red were ineffective when added alone (Figure 1a), they brought about pore closure when added together (Figure 1b). It is clear that CSA and Ruthenium Red induce pore closure in a synergistic manner.

Induction of CSA blockade by cobaltammines, inhibitors of the Ca^{2+} uniporter

Since Ruthenium Red blocks the uptake of Ca^{2+} by mitochondria that is mediated by the Ca^{2+} uniporter, the obvious question was whether uniporter inhibition was the relevant factor in the synergism. This question was addressed using a range of cobaltammine complexes as inhibitors of the uniporter. Ruthenium Red itself was not used for two reasons. First, it seems that uniporter inhibition by commercially supplied Ruthenium Red is not due to Ruthenium Red itself (i.e. $[A_5Ru\text{-}O\text{-}Ru(A_4)O\text{-}RuA_5]^{6+}$), but to a minor contaminant, the dinuclear ruthenium ammine complex $[A_4(Cl)Ru\text{-}O\text{-}Ru(Cl)A_4]^{3+}$ [21]; this being so, it could not be ruled out that the observed effects on $\Delta\psi$ (Figure 1b) might be caused by other components of the commercially supplied samples. Secondly, Ruthenium Red and its inhibitory

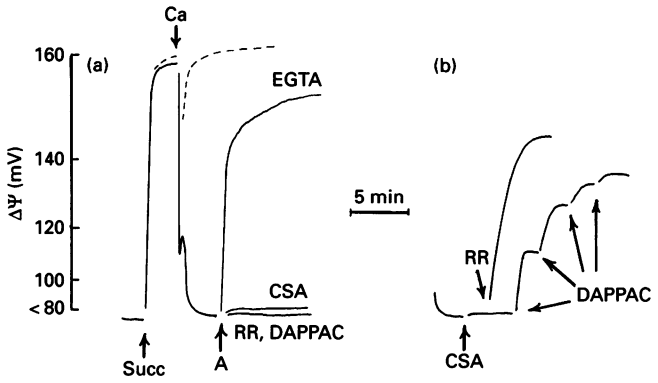

Figure 1 Ca^{2+}-induced collapse of $\Delta\psi$ and its restoration by CSA, Ruthenium Red and diaminopentanepentamminecobalt

(**a**) Respiration was started with 5 mm succinate (Succ) in the absence (solid lines) and presence (broken line) of 1 μM CSA. Further additions were 30 nmol of Ca^{2+}/mg of mitochondrial protein (Ca) and, at arrow A, 1 mM EGTA, 1 μM CSA, 1 μM Ruthenium Red (RR) or 10 μM $[Co(diaminopentane)A_5]^{4+}$ (DAPPAC). (**b**) $\Delta\psi$ was generated (succinate) and then collapsed (Ca) as in (**a**) (not shown). Further additions were 1 μM CSA, 1 μM Ruthenium Red (RR) and 1.2 μM additions (at each arrow) of $[Co(diaminopentane)A_5]^{4+}$ (DAPPAC).

Table 1 Potency of the cobaltammine complexes as uniporter inhibitors

For each complex the concentration producing 50% inhibition of uniporter activity was determined with three mitochondrial preparations (all at 1 mg of protein/ml). For three of the cobaltammines, data are given as means ± S.E.M.

Cobaltammine	Concentration producing 50% inhibition (μM)
$[Co(NH_3)_6]^{3+}$	1.66 ± 0.37
$[Co(H_2O)(NH_3)_5]^{3+}$	0.54 ± 0.18
$[Co(NH_2CH_2CH_2CH_2CH_2CH_2NH_3)(NH_3)_5]^{4+}$	0.14 ± 0.04
$[Co(NH_3CH_2CH_2COO)(NH_3)_5]^{3+}$	> 10

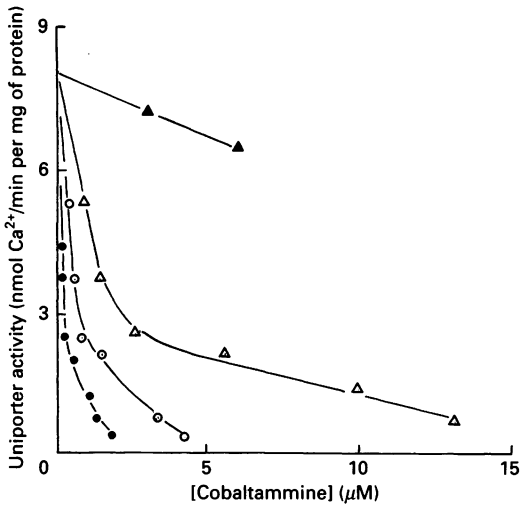

Figure 2 Inhibition of the Ca^{2+} uniporter by cobaltammine complexes

Uniporter activity was measured as described in the Materials and methods section. ●, $[Co(diaminopentane)A_5]^{4}$; ○, $[Co(H_2O)A_5]^{3+}$; △, $[CoA_6]^{3+}$; ▲, $[Co(Ala)A_5]^{3+}$.

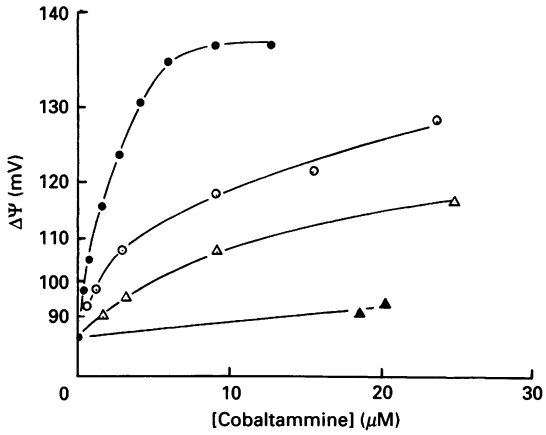

Figure 3 Dependence of the restoration of $\Delta\psi$ by CSA on the concentration of cobaltammine

$\Delta\psi$ was measured as in Figure 1(b) in the presence of 1 μM CSA and various concentrations of cobaltammine: ●, $[Co(diaminopentane)A_5]^{4+}$; ○, $[Co(H_2O)A_5]^{3+}$; △, $[CoA_6]^{3+}$; ▲, $[Co(Ala)A_5]^{3+}$.

component adhere tenaciously to glassware and plastics, making it difficult to draw comparisons between different incubations (vessels).

The cobaltammine complexes investigated are listed in Table 1; their effects on uniporter activity are illustrated in Figure 2. It is important to point out that all Ca^{2+} influx rates were corrected for the slow rates of Ca^{2+} efflux (see the Materials and methods section; [15]). In addition, it was confirmed that in the absence of Ca^{2+} (1 mM EGTA), none of the cobaltammines had any detectable effect (over the concentration range reported) on the magnitude of $\Delta\psi$, the driving force for Ca^{2+} accumulation. Thus true uniporter activities were measured. All of the cobaltammine ions inhibited the uniporter, but with different affinities (Figure 2), i.e. $[Co(pentanediamine)A_5]^{4+} > [Co(H_2O)A_5]^{3+} > [CoA_6]^{3+}$

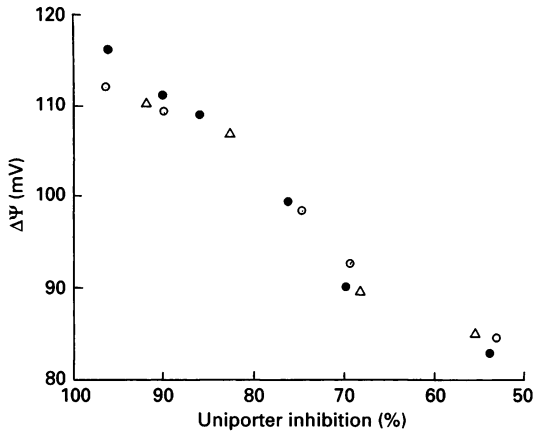

Figure 4 Relationship between $\Delta\psi$ restoration and the degree of uniporter inhibition by three cobaltammines

The re-establishment of $\Delta\psi$ (as in Figure 3) and the degree of uniporter inhibition (as in Figure 2) were measured in a single population of mitochondria for the following cobaltammine complexes: ●, $[Co(diaminopentane)A_5]^{4+}$; ○, $[Co(H_2O)A_5]^{3+}$; △, $[CoA_6]^{3+}$.

$\gg [Co(Ala)A_5]^{3+}$. The cobaltammine concentrations that produced 50% inhibition of the uniporter in three independent experiments are summarized in Table 1.

We investigated whether the cobaltammines enabled CSA-dependent restoration of $\Delta\psi$, as reported in Figure 1 for $[Co(pentanediamine)A_5]^{4+}$. It is evident that the cobaltammine promoted the regeneration of $\Delta\psi$ in a concentration-dependent manner in the presence of CSA (Figure 1b), while it was ineffective in the absence of CSA (Figure 1a). This behaviour was observed for all the cobaltammine complexes, although the effective range of cobaltammine concentrations varied. Typical data for the relationship between $\Delta\psi$ and the concentrations of the four cobaltammine ions in the presence of CSA are reported in Figure 3. The order of effectiveness in restoring $\Delta\psi$ was $[Co(pentanediamine)A_5]^{4+} > [Co(H_2O)A_5]^{3+} > [CoA_6]^{3+} \gg [Co(Ala)A_5]^{3+}$. This order is the same as that for uniporter inhibition.

The broad correlation between the capacities of the cobaltammines to inhibit the uniporter and to facilitate CSA inhibition of the pore was examined more quantitatively by comparing the relationship between $\Delta\psi$ and the degree of inhibition of the uniporter for three of the cobaltammines. ($[Co(Ala)A_5]^{3+}$ was not included since its ability to inhibit the uniporter and to restore $\Delta\psi$ was extremely low.) Figure 4 shows that the curves representing this relationship for the three cobaltammines were essentially superimposable. This was in spite of the fact that the three cobaltammine ions exhibited quite different affinities for the uniporter. This correspondence provides good evidence that the cobaltammine-induced pore blockade was due to uniporter inhibition, rather than a direct effect of the cobaltammine on the pore itself.

Ca^{2+}-induced suppression of CSA binding and its reversal by cobaltammines and Ruthenium Red

Cobaltammine inhibition of the uniporter would lead to a decrease in mitochondrial Ca^{2+}. Accordingly, we considered the possibility that a high intramitochondrial Ca^{2+} concentration might oppose CSA inhibition of the pore, and that the action of the cobaltammines in facilitating pore inhibition by CSA lay in the fact that they decreased mitochondrial Ca^{2+} accumulation. This was tested by measuring the binding of [^{3}H]CSA to respiring

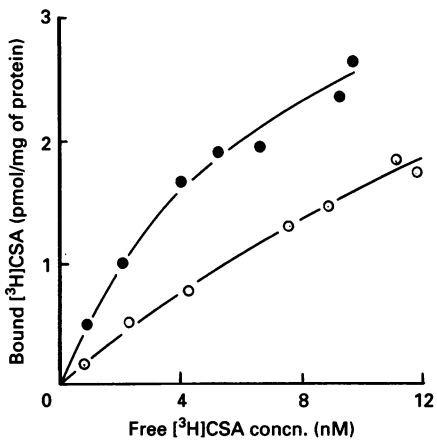

Figure 5 Influence of Ca^{2+} on CSA binding to mitochondria

[^{3}H]CSA binding was measured as stated (see the Materials and methods section) in the presence (○) and absence (●; 1 mM EGTA) of 25 nmol of Ca^{2+}/mg of mitochondrial protein.

Table 2 Reversibility of Ca^{2+}-sensitive [^{3}H]CSA binding

[^{3}H]CSA binding was measured with a fixed amount of [^{3}H]CSA (approx. 7 nM free [^{3}H]CSA) as described in the Materials and methods section, except that modifications were made as stated under 'Protocol'. For example, in protocol 1, mitochondria were incubated with 25 μM EGTA for 3 min; [^{3}H]CSA was then added and binding was measured 8 min later. All data were obtained with the same mitochondrial preparation and are given as means ± S.E.M. ($n = 6$). * $P < 0.002$ compared with protocol 1.

Protocol no.	Protocol	[^{3}H]CSA bound (pmol/mg of protein)
1	(i) 25 μM EGTA (3 min) (ii) [^{3}H]CSA (8 min) (iii) Measure	3.51 ± 0.18
2	(i) 25 μM Ca^{2+} (3 min) (ii) [^{3}H]CSA (8 min) (iii) Measure	2.04 ± 0.06*
3	(i) 25 μM EGTA (3 min) (ii) [^{3}H]CSA (8 min) (iii) 50 μM Ca^{2+} (3 min) (iv) Measure	1.93 ± 0.11*

mitochondria in the presence and absence of Ca^{2+}. The concentrations of free [^{3}H]CSA used were within the range over which pore inhibition is produced (i.e. 0–30 nM; [14]). The data (Figure 5) reveal that [^{3}H]CSA binding was indeed markedly inhibited by Ca^{2+}. Further experiments (results not shown) confirmed that an increase in Ca^{2+} (from 25 μM to 60 μM) did not decrease [^{3}H]CSA binding further, indicating that in Figure 5 maximal Ca^{2+} inhibition was attained. This does not necessarily mean that Ca^{2+} inhibits CSA binding to the relevant component only partially, since liver mitochondria may contain two high-affinity CSA binding components [14], only one of which may be sensitive to Ca^{2+}. In other words, the residual [^{3}H]CSA binding in the presence of Ca^{2+} may reflect a Ca^{2+}-insensitive component(s).

It was also relevant to check the reversibility of [^{3}H]CSA binding to the Ca^{2+}-sensitive component. In the experiment of Table 2 the binding in the presence of EGTA (protocol 1) was

Table 3 Reversal by cobaltammines and Ruthenium Red of the Ca^{2+}-induced suppression of CSA binding

[^{3}H]CSA binding was measured with a fixed amount of [^{3}H]CSA (yielding about 5 nM free [^{3}H]CSA), as in Figure 5. The following additions were made 2 min before [^{3}H]CSA as indicated: 1 μM Ruthenium Red, 10 μM $[Co(diaminopentane)A_5]^{4+}$, 50 μM $[Co(H_2O)A_5]^{3+}$, 1 mM EGTA and 25 μM Ca^{2+}. The data are means ± S.E.M. ($n = 6$) with a single mitochondrial preparation. * $P < 0.002$ compared with addition of Ca^{2+} only.

Addition(s)	[^{3}H]CSA bound (pmol//mg of protein)
EGTA	2.20 ± 0.13*
Ca^{2+}	1.45 ± 0.09
Ruthenium Red, Ca^{2+}	2.22 ± 0.19*
$[Co(diaminopentane)A_5]^{4+}$, Ca^{2+}	2.19 ± 0.08*
$[Co(H_2O)A_5]^{3+}$, Ca^{2+}	2.17 ± 0.22*

decreased by 42% in the presence of Ca^{2+} (protocol 2), and a similar decrease (45%) occurred when Ca^{2+} was introduced after preincubation with EGTA (protocol 3). It may be concluded, therefore, that Ca^{2+} not only inhibits [^{3}H]CSA binding but also induces the release of bound [^{3}H]CSA.

Further studies (Table 3) revealed that the suppression of CSA binding brought about by Ca^{2+} was abolished by [Co(pentanediamine)A_5]$^{3+}$, $[Co(H_2O)A_5]^{3+}$ and Ruthenium Red; $[CoA_6]^{3+}$ (at 50 μM) also blocked the action of Ca^{2+} (results not shown). The most straightforward interpretation is that CSA binding is antagonized by Ca^{2+} in the intramitochondrial compartment, so that Ca^{2+} becomes ineffective when its accumulation is blocked. By itself, this does not necessarily imply that Ca^{2+} antagonizes CSA binding to a component of the pore; in principle, CSA binding to some other mitochondrial protein may have been affected. However, when these data are taken together with the fact that inhibition of the pore by CSA is promoted by inhibitors of mitochondrial Ca^{2+} uptake (Figures 3 and 4), it seems most likely that CSA binding to a pore component was indeed affected.

DISCUSSION

It was known that intramitochondrial Ca^{2+} ($K_d > 20$ μM) is responsible for pore activation [19], but not whether internal Ca^{2+} has any influence on pore interaction with its inhibitor CSA. Although this question might be approached by varying the extramitochondrial Ca^{2+}, so that intramitochondrial Ca^{2+} varies too, this would not distinguish between the effects of internal and external Ca^{2+}; it has been suggested that the pore contains both internal and external binding sites for bivalent cations [22]. The use of a single inhibitor of Ca^{2+} uptake would also be ambiguous, since one would need to discriminate between the consequences of lowered intramitochondrial Ca^{2+} and direct effects of the inhibitor on the pore itself. In the present study we have attempted to circumvent these problems by using several uniporter inhibitors with a range of affinities. Because of their ease of synthesis, cobaltammine complexes were tried and found to be satisfactory. The fact that the cobaltammines promoted inhibition of the pore by CSA strictly according to their potential as uniporter inhibitors (Figures 2–4) provides firm evidence that increases in intramitochondrial Ca^{2+} diminish the ability of CSA to inhibit the pore. Regarding the underlying mechanism, the findings that CSA binding is suppressed by Ca^{2+} (Figure 5), and that this suppression is in turn blocked when Ca^{2+} uptake is prevented (Table 3), strongly indicate that high intramitochondrial Ca^{2+} antagonizes the binding of CSA to the relevant component of the pore.

As yet there is no consensus over the amount of the component that confers CSA-sensitivity to the pore. Whereas measurements using [^{3}H]CSA suggested a value of < 6 pmol/mg of mitochondrial protein [14], other techniques indicated considerably more (> 50 pmol/mg [13]). It should also be pointed out that there is a further discrepancy between the amounts of [^{3}H]CSA bound in this and previous studies (e.g. approx. 2 pmol/mg of protein at 5 nM free [^{3}H]CSA [14]) and the amounts that would be predicted from other investigations showing that liver mitochondria contain about 50 pmol of cyclophilin/mg of mitochondrial protein [13] with a K_i value for CSA of 4–8 nM [13,14] (from which at least 20–30 pmol of bound CSA would be predicted with 5 nM free CSA). Although further studies are required to resolve the nature of these differences, it remains possible that a large proportion of intramitochondrial cyclophilin may be screened from binding CSA by combination with other matrix constituents.

The conclusion that Ca^{2+} inhibits CSA binding bears on three aspects of current interest. First, the loss of the effectiveness of CSA as a pore inhibitor (e.g. Figure 1) has been the subject of studies by Novogorodov et al. [20], who have shown that pore sensitivity is restored by adenine nucleotides. Adenine nucleotides inhibit Ca^{2+}-dependent pore opening [3,6]. In other words, whereas inhibition of the pore by CSA is promoted by adenine nucleotide inhibitors, it is diminished by Ca^{2+}, the key activator of the pore (the present paper). Thus it is conceivable that adenine nucleotides act by decreasing the affinity of the system for Ca^{2+} and thereby not only prevent pore activation but also facilitate its interaction with CSA. Secondly, direct measurements of pore state ([^{14}C]sucrose permeation) have shown that CSA does not inhibit the pore completely (e.g. Figure 2 in [14]). The present data provide a possible explanation, since CSA-induced pore closure and regeneration of $\Delta\psi$ would be counteracted by subsequent re-accumulation of Ca^{2+} and displacement of bound CSA. One would anticipate a steady-state balance between these opposing effects and, therefore, a certain fraction of pores in the open state at any point in time. Thirdly, and most importantly, the fact that CSA binding is antagonized by Ca^{2+} may find practical application in identifying the particular protein that confers CSA sensitivity to the pore. Although there is a case for the involvement of cyclophilin [4,13,14], [^{3}H]CSA binds with high affinity to more than one mitochochondrial protein [14], and the question which of these (in intact mitochondria at least) binds CSA in a Ca^{2+}-inhibitable manner would seem to be most relevant.

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