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Sanglifehrin A Acts as a Potent Inhibitor of the Mitochondrial Permeability Transition and Reperfusion Injury of the Heart by Binding to Cyclophilin-D at a Different Site from Cyclosporin A*

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Cyclosporin A (CsA) inhibits opening of the mitochondrial permeability transition pore (MPTP), a critical event in some forms of necrotic and apoptotic cell death, by binding to cyclophilin D (CyP-D) and inhibiting its peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. Sanglifehrin A (SfA), like CsA, exerts its immunosuppressive action by binding to cyclophilin A but at a different site from CsA, and unlike the latter, SfA does not inhibit calcineurin activity. Here we demonstrate that SfA inhibits the PPIase activity of CyP-D ($K_{0.5}$ 2 nM) and acts as a potent inhibitor of MPTP opening under both energized and de-energized conditions. However, unlike CsA, the dose-response curve for inhibition by SfA is sigmoidal rather than hyperbolic, suggesting a multimeric structure for the MPTP with cooperativity between subunits. Furthermore, SfA does not prevent CyP-D binding to submitochondrial particles or detergent-solubilized adenine nucleotide translocase (ANT), implying that CyP-D binding to the ANT does not require PPIase activity but pore opening does. Once bound to the MPTP, SfA is not readily dissociated, and inhibition of pore opening is maintained following extensive washing. To investigate the potential of SfA as an inhibitor of cell death *in vivo*, we used the Langendorff perfused rat heart. SfA caused a time-dependent inhibition of the MPTP that was maintained on mitochondrial isolation to a greater extent than was CsA inhibition. We demonstrate that SfA, like CsA, improves the recovery of left ventricular developed pressure during reperfusion after 30 min of global ischemia and greatly reduces lactate dehydrogenase release, implying inhibition of necrotic damage. Because SfA does not inhibit calcineurin activity, our data suggest that it may be more desirable than CsA for protecting tissues recovering from ischemic episodes and for studying the role of the MPTP in cell death.

A critical event in some forms of necrotic and apoptotic cell death is the opening of the mitochondrial permeability transition pore (MPTP)[1] (1–4), the formation of which is widely thought to involve an interaction between the adenine nucleotide translocase (ANT) and cyclophilin D (CyP-D (1, 2). To date, the most specific inhibitor of the MPTP is cyclosporin A (CsA) (5), which acts by inhibiting the peptidyl-prolyl *cis-trans* isomerase (PPIase) activity of CyP-D (6, 7). Indeed, CsA can act as a potent inhibitor of both apoptotic and necrotic cell death under some circumstances (1–3). Nevertheless, inhibition of cell death by CsA does not provide definitive evidence for a critical role of the MPTP in the process, because CsA exerts other effects on cell function. In particular, the immunosuppressant action of the drug is mediated through inhibition of calcineurin, a calcium-activated protein phosphatase involved in the regulation of gene expression and other intracellular functions (8–10). However, there are CsA analogues such as *N*-Me-Ala-6-cyclosporin A and *N*-Me-Val-4-cyclosporin A that do not inhibit calcineurin activity and yet still inhibit the PPIase activity of CyP-D, antagonize the opening of the MPTP and protect cells from apoptotic and necrotic cell death (11–13). In this paper we investigate another immunosuppressant drug, sanglifehrin A, that is unrelated to CsA but like CsA binds tightly to cyclophilin A (CyP-A) with a $K_{0.5}$ of 4–7 nM, inhibiting its PPIase activity ($K_{0.5}$ 13 nM). However, unlike the CyP-A·CsA complex, the CyP-A·SfA complex does not inhibit calcineurin (14, 15). Rather, SfA specifically blocks T cell proliferation in response to interleukin 2 through a mechanism involving an NFκB-mediated increase in the expression of the tumor suppressor genes *p53* and *p21*, with the latter binding to and inhibiting the appearance of cell cycle kinase activity cyclin E-Cdk2 (14, 16, 17).

If SfA were able to bind tightly to CyP-D and inhibit its PPIase activity and the opening of the MPTP, its lack of effect on calcineurin might make it an alternative to *N*-Me-Ala-6-cyclosporin A and *N*-Me-Val-4-cyclosporin A in studying the role of the MPTP in cell death. Here, we demonstrate that SfA is as potent as CsA as an inhibitor of the PPIase activity of CyP-D and of MPTP opening. It is also able to improve functional recovery of rat hearts reperfused following an ischemic episode, as well as reduce necrotic damage. However, SfA shows some important differences from CsA in its mode of action on the MPTP. It demonstrates a sigmoidal rather than a hyperbolic dose-response curve for MPTP inhibition and does not prevent CyP-D binding to the ANT. These data provide new insights into the molecular mechanism of the MPTP.

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[1] The abbreviations used are: MPTP, mitochondrial permeability transition pore; ANT, adenine nucleotide translocase; CyP, cyclophilin; CsA, cyclosporin A; LVDP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; PEG, polyethylene glycol; PPIase, peptidyl-prolyl *cis-trans* isomerase; SfA, sanglifehrin A; SMP, submitochondrial particle; GST, glutathione *S*-transferase; Mops, 4-morpholinepropanesulfonic acid.

EXPERIMENTAL PROCEDURES

Preparation of Mitochondria and Submitochondrial Particles (SMPs)—Percoll-purified rat liver mitochondria and SMPs were prepared as described previously (18, 19). For measurement of MPTP opening under de-energized conditions, they were stored on ice overnight before use to deplete them of adenine nucleotides (18).

MPTP Opening—The opening of the MPTP was determined under energized or de-energized conditions by following the decrease in light scattering (monitored as A_{520}) that accompanies mitochondrial swelling at 25 °C (6). The buffer for energized conditions was 125 mM KCl, 20 mM Mops, 10 mM Tris, 2 mM potassium P_i, 2 mM succinate, 0.5 μM rotenone, pH 7.2, and for de-energized conditions was 150 mM KSCN, 20 mM Mops, 10 mM Tris, 2 mM nitrilotriacetic acid, 2 μM A23187, 0.5 μM rotenone, 0.5 μM antimycin A, pH 7.2. Additions of CsA (Calbiochem) or SfA (Novartis) were made as required. MPTP opening was initiated by addition of $CaCl_2$ to give the free [Ca^{2+}] indicated. The sensitivity of the MPTP to [Ca^{2+}] was also determined by following the shrinkage of pre-swollen mitochondria (as an increase in A_{520}) upon the addition of polyethylene glycol (PEG-2000) as described previously (18, 20). De-energized buffer was used, and pre-swollen mitochondria were preincubated for 2 min with the required [Ca^{2+}] and other additions before initiation of shrinkage by the addition of 7% (w/v) PEG. The rates of swelling and shrinking of mitochondria were determined by differentiation of the A_{520} time course (18).

PPIase Activity of CyP-D—The PPIase activity of recombinant CyP-D (produced by thrombin cleavage of GST-CyP-D (21)) was determined by following the rate of hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide by chymotrypsin as described previously (6, 7). Chymotrypsin only hydrolyzes the *trans* form of the peptide, and hydrolysis of the *cis* form, the concentration of which is maximized by using a stock dissolved in trifluoroethanol containing 470 mM LiCl (22), is limited by the rate of *cis-trans* isomerization.

CyP-D Binding to SMPs—Recombinant CyP-D (10 μg), prepared as described previously (21), was added to 100 μg of SMPs (4 mg protein/ml in isolation medium) and incubated for 15 min at 22 °C before sedimenting by centrifugation (157,000 × *g* for 5 min at 4 °C). After being washed once in isolation buffer, bound CyP-D was determined by SDS-PAGE and Western blotting with anti-CyP-D antibodies (21).

Binding of ANT to GST-CyP-D—This was performed essentially as described previously using detergent-solubilized purified inner mitochondrial membrane and recombinant GST-CyP-D bound to glutathione-Sepharose (21). When required, the bound GST-CyP-D was preincubated with CsA or SfA (25 μM) for 15 min at 22 °C prior to the addition of solubilized inner mitochondrial membranes. After extensive washing the specifically bound protein was then eluted with glutathione and analyzed by SDS-PAGE and Western blotting with anti-ANT antibodies (21).

Heart Perfusion—Isolated rat hearts were perfused in the Langendorff mode as described previously (23) with continuous monitoring of left ventricular developed pressure (LVDP) and left ventricular end diastolic pressure (LVEDP) using a pressure transducer connected to a water-filled balloon inserted into the left ventricle. Following control perfusion for 50 min, with the addition of CsA or SfA as required, perfusion was halted to initiate global isothermic (37 °C) ischemia. After 30 min, ischemia perfusion was restarted and was continued for 10 min in the presence of drug and a further 20 min in its absence. Samples of perfusate were collected prior to ischemia and every 1 min during reperfusion for the determination of lactate dehydrogenase activity spectrophotometrically.

RESULTS

SfA Inhibits the PPIase Activity of CyP-D and MPTP Opening—In Fig. 1 we show that SfA inhibits the PPIase activity of recombinant CyP-D with a K_i (±S.E.) of 2.2 ± 0.7 nM, a potency similar to that of CsA (6, 7). In Fig. 2*a* we demonstrate that submicromolar concentrations of SfA inhibit opening of the MPTP of energized mitochondria induced by 100 nmol of Ca^{2+}/mg of mitochondrial protein. However, the concentration dependence of inhibition was complex, with the lowest concentration of SfA (100 nM) failing to exhibit any inhibition no matter how long the mitochondria were exposed to the drug. We have previously shown (18) that energized mitochondria are not ideal for probing the mechanism of the MPTP because many factors can affect the sensitivity of pore opening to [Ca^{2+}]. These include changes in membrane potential, matrix adenine nucleotide concentrations, and calcium transport (18). In addition, the accumulation of calcium in the presence of phosphate can lead to an increase in light scattering prior to pore opening that becomes dominant when the MPTP is inhibited (6, 24–28). This is apparent in Fig 2*a*. For this reason, we routinely use de-energized conditions (iso-osmotic KSCN buffer with respiratory chain inhibitors) in the presence of the calcium ionophore A23187 to ensure that calcium equilibrates across the inner mitochondrial membrane. Mitochondria are also stored overnight following their preparation because they lose a fair proportion of their adenine nucleotides during the first few hours of storage on ice, which causes the sensitivity of the MPTP toward calcium to change over this period. Using these conditions it is possible to obtain detailed dose-response curves for CsA analogues allowing calculation of $K_{0.5}$ values that closely match the K_i for inhibition of the PPIase activity of CyP-D (6, 7).

The data of Fig. 2*b* show some typical traces for the inhibition of the MPTP by CsA and SfA under such de-energized conditions, whereas in Fig. 3 we present detailed dose-response curves. These data show clearly that CsA and SfA behave differently. Whereas inhibition by CsA is progressive and can be fitted to the equation for binding to a single site as described previously (6, 7), the inhibition by SfA is sigmoidal with little or no inhibition apparent at low inhibitor concentrations. However, a steep concentration dependence for inhibition developed as SfA reached a concentration at which CsA gave about 50% inhibition, the value being dependent on the concentration of mitochondria in the assay (Fig. 3*b*). The concentration of SfA required to inhibit pore opening by >80% was almost identical to that when CsA was used (Fig 3*a*), consistent with both inhibitors associating with the same target protein (CyP-D). SfA also inhibited the MPTP when assayed using the shrinkage assay, and here too the concentration dependence for inhibition was sigmoidal as shown in Fig. 4*a*. The effect of SfA, like that by CsA (18), was to decrease the sensitivity of the MPTP to [Ca^{2+}] as shown in Fig. 4*b*. Thus, even in the presence of a concentration of SfA that gave maximal inhibition (1 μM), the pore could be opened at higher [Ca^{2+}].

Distinct Effects of SfA and CsA on CyP-D Binding to SMPs and Solubilized ANT—The effects of SfA and CsA on CyP-D binding to SMPs were compared, and the data are shown in Fig. 5*a*. SfA slightly increased the binding of recombinant CyP-D to SMPs (*lane 4*), unlike CsA, which greatly reduced binding (*lane 3*) as observed previously (12, 20). In nine such experiments the mean binding of CyP-D in the presence of SfA as percentage of control (±S.E.) was 132 ± 12% ($p < 0.05$), whereas for CsA the binding was reduced to 34 ± 8% ($p < 0.001$). Treatment of the CyP-D with SfA before (*lane 7*) or after (*lane 6*) CsA treatment overcame the CsA inhibition of CyP-D binding. A slight reversal of CyP-D binding to SMPs was detected when CsA was added after CyP-D was already bound (*lane 8*), but again no effect of SfA was observed (*lane 9*). These data suggest that CsA binds to CyP-D at the same site as the ANT, whereas SfA binds to a distinct site that actually enhances CyP-D binding to the ANT while displacing bound CsA. Further evidence in favor of this interpretation was obtained when the effects of CsA and SfA on the binding of ANT present in detergent-solubilized inner mitochondrial membranes to a GST-CyP-D affinity column were studied (Fig. 5*b*). ANT binding was enhanced by pretreatment of mitochondria with diamide and inhibited by pretreatment of the GST-CyP-D with CsA as found previously (21). In contrast, SfA did not prevent binding, and in the case of diamide-treated mitochondria, the binding of ANT was actually increased by SfA.

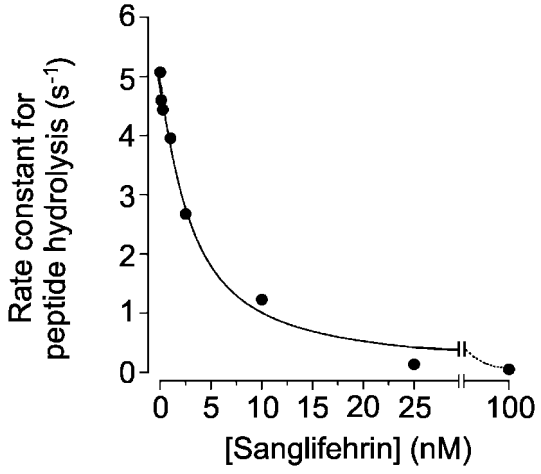

FIG. 1. **Inhibition of PPIase activity of CyP-D by SfA.** Recombinant CyP-D (6 pmol) was preincubated at 10 °C for 5 min in 3.5 ml of PPIase assay buffer (see "Experimental Procedures") with the concentrations of SfA shown. The assay was started by the addition of peptide, and initial rates of reaction were determined by first order regression analysis of the time course of change in A_{520}. The K_i (±S.E.) derived by fitting the data to the equation for a tight binding inhibitor (6) was 2.2 ± 0.7 nM.

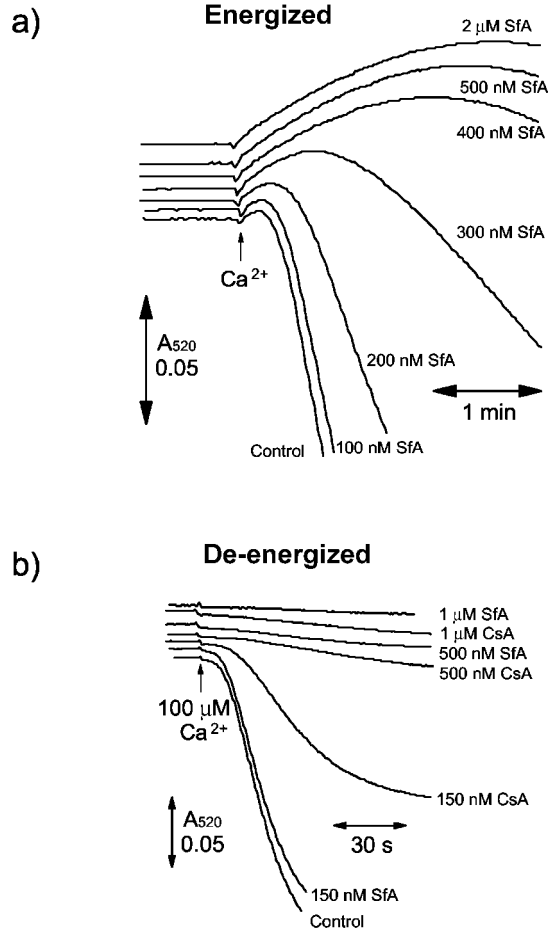

FIG. 2. **Inhibition of the MPTP by SfA.** In *panel a*, MPTP opening in rat liver mitochondria (1.5 mg protein/ml) was assayed under energized conditions (see "Experimental Procedures") by monitoring the decrease in A_{520} with a split beam spectrophotometer after the addition of 100 μM $CaCl_2$ to the sample cuvette. When present, SfA or CsA were added at the concentrations shown at 2 min before calcium addition. In *panel b* the protocol was similar, but de-energized conditions were employed and pore opening initiated by the addition of $CaCl_2$ to give a calculated free concentration of 100 μM.

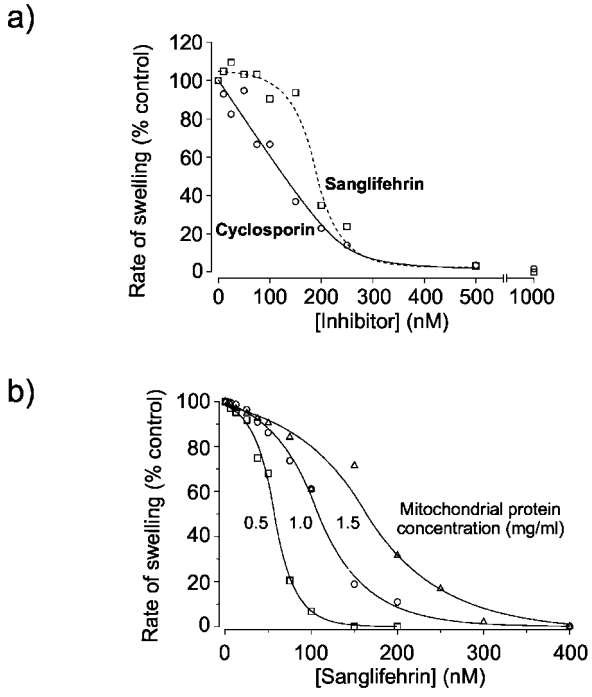

FIG. 3. **Concentration dependence of the inhibition of the MPTP by SfA.** In *both panels* the extent of MPTP opening was determined from the rate of swelling in experiments similar to those shown in Fig 2*b* but with CsA or SfA added 2 min before $CaCl_2$ at the concentrations indicated. In *panel a* the protein concentration was 1.5 mg/ml, whereas in *panel b* the concentration of mitochondria was varied as indicated.

SfA Protects the Ischemic Heart from Reperfusion Injury—The MPTP has now been recognized as playing a critical role in both necrotic and apoptotic cell death, and evidence for this has come from the ability of CsA to protect cells from a variety of death signals (1–3). We and others (23, 29, 30) have demonstrated that the necrotic damage associated with reperfusion of the ischemic rat heart (irreversible reperfusion injury) can be protected by pretreatment of the heart with CsA, with optimal effects being observed at 0.2 μM. Thus it would be expected that SfA might act in the same manner. Initial experiments using Langendorff perfused rat hearts subjected to 30 min of global ischemia followed by reperfusion failed to show any protective effects of 0.2 μM SfA when functional recovery was determined by measurement of LVDP and LVEDP. One explanation of why SfA gave no protection under these conditions would be that it fails to reach the mitochondria in the perfused heart. To test this possibility the experiments reported in Fig. 6 were performed.

In Fig. 6*a* we investigated whether, following treatment of mitochondria with SfA, the drug remains bound to CyP-D through extensive washing of mitochondria in the absence of SfA. Mitochondria pretreated with 1 μM CsA and then washed free of CsA exhibited greatly reduced MPTP opening in response to Ca^{2+} when compared with untreated mitochondria, although the inhibition was not as complete as when the CsA was added directly to the assay. These data suggest that some CsA dissociated from CyP-D during the washing. In contrast, mitochondria pretreated with 1 μM SfA maintained complete inhibition of the MPTP following washing. These data indicate that SfA dissociates from CyP-D less readily than CsA. In addition, they imply that if SfA has access to mitochondria within the perfused heart, this will be detected as an inhibition of the MPTP in subsequently isolated mitochondria. In Fig. 6*b* we demonstrate that this is the case.

In hearts pretreated with 0.2 μM CsA for 3 min, the condi-

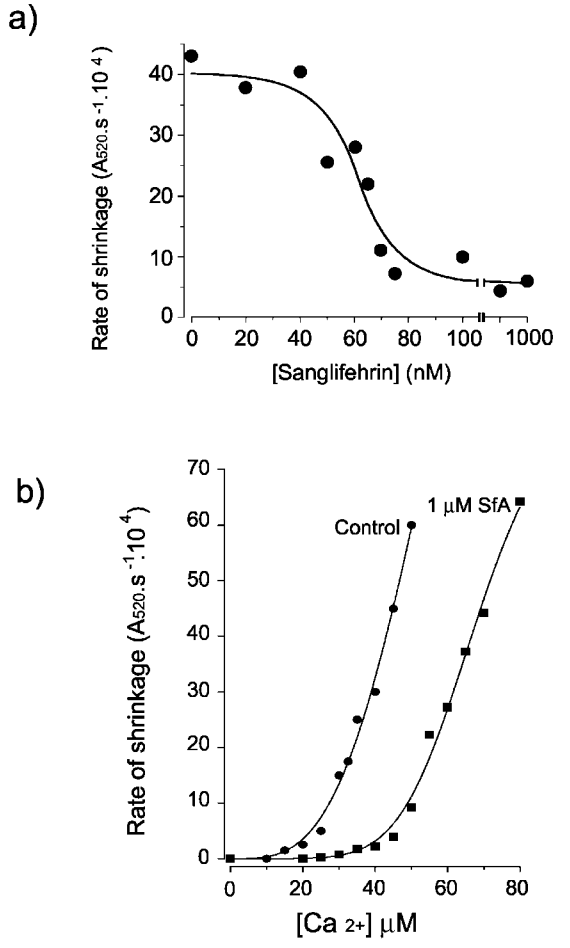

FIG. 4. **The effect of SfA on the calcium dependence of the MPTP.** In *both panels* the extent of MPTP opening was determined by measuring the rate of shrinkage of pre-swollen mitochondria upon the addition of 7% (w/v) PEG as described under "Experimental Procedures." In *panel a* the pre-swollen mitochondria were incubated for 2 min with 50 μM [Ca^{2+}] and the concentration of SfA shown before the addition of PEG to initiate shrinkage. In *panel b* the concentration of Ca^{2+} was varied as indicated in the absence or presence of 1 μM SfA.

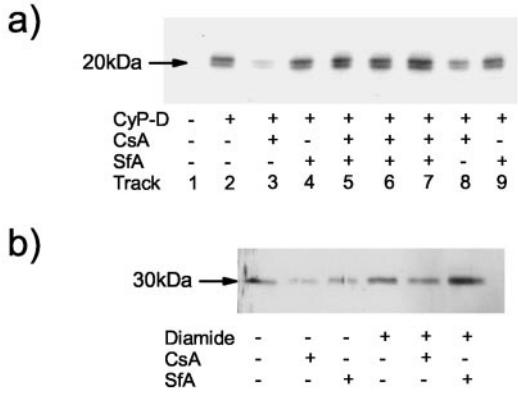

FIG. 5. **CsA but not SfA prevents CyP-D binding to SMPs and ANT binding to GST-Cyp-D.** In *panel a*, recombinant CyP-D (10 μg) was preincubated for 15 min at 22 °C with 50 μM CsA, 50 μM SfA, or solvent (ethanol) as indicated before the addition of SMPs. After incubation for a further 15 min at 22 °C, bound CyP-D was determined by SDS-PAGE and Western blotting as described under "Experimental Procedures." In *lane 5*, SfA and CsA were added together. In *lane 6*, CyP-D was incubated with CsA for 15 min followed by SfA for 15 min, whereas in *lane 7*, the order of addition of the inhibitors was reversed. In *lanes 8* and *9*, the CsA and SfA were added after incubation of SMPs with CyP-D, and incubation was continued for a further 15 min. In *panel b*, inner mitochondrial membranes were prepared from rat liver mitochondria after incubation in the presence or absence of 1 mM diamide for 10 min at 22 °C. After detergent solubilization, they were passed down a GST-CyP-D affinity column that had been preincubated with or without 25 μM CsA or SfA as described under "Experimental Procedures." Following extensive washing, bound proteins were eluted with glutathione and analyzed by SDS-PAGE and Western blotting with anti-ANT antibodies.

tions found optimal for protecting hearts from reperfusion injury (23), some inhibition of MPTP opening was detected at both 100 and 200 μM Ca^{2+}. In contrast, treatment with 0.2 μM SfA for 5 min gave no detectable inhibition of the MPTP, consistent with its inability to protect hearts from reperfusion injury under these conditions. However, when hearts were perfused with 1 μM SfA for 10 min, almost total inhibition of the MPTP could be demonstrated. This inhibition is unlikely to be due to the carry over of SfA into the isolation buffer because the total amount of extracellular medium in the heart (about 0.5 ml) contains only 500 pmol of SfA. This compares with a mitochondrial CyP-D content of the heart of >2000 pmol (calculated from data in Refs. 11 and 31). However, we have confirmed this conclusion experimentally in two ways. First, when 1 μM SfA was perfused through the heart for only 1 min prior to mitochondrial preparation, opening of the MPTP was similar to control mitochondria (data not shown). Second, when hearts were exposed to 1 μM SfA for 10 min and then the SfA removed by a 30-s perfusion with SfA-free buffer, the MPTP opening in subsequently isolated mitochondria remained inhibited even at 1 mM calcium (Fig. 6*b*). Thus it would be predicted that these conditions should protect hearts from reperfusion injury; the data of Fig. 7 demonstrate that this is indeed the case.

In Fig. 7*a* we show that pretreatment of hearts with 0.2 μM CsA for 3 min before 30 min of global ischemia greatly improved function recovery of the heart during reperfusion, as reflected in the greater LVDP (69 ± 8% of pre-ischemic values, *cf.* control value of 26 ± 8%; $p < 0.01$) and lower LVEDP (37 ± 6 mm Hg, *cf.* control value of 64 ± 6; $p < 0.01$). These data confirm previous data from our and other laboratories (23, 30). Very similar results were obtained when hearts were pretreated with 1 μM SfA for 10 min, with recovery of LVDP being 79 ± 10% of pre-ischemic values and the LVEDP being 33 ± 4 mm Hg (both $p < 0.01$ *versus* controls). To confirm that this functional protection reflects an inhibition of necrotic cell death during reperfusion, lactate dehydrogenase release was determined, and the results are reported in Fig. 7*b*. It is clear that both SfA and CsA greatly reduce lactate dehydrogenase release coincident with their protection of heart function.

DISCUSSION

The Molecular Mechanism of the MPTP—We have shown previously (6, 7, 18, 20, 21, 32) and confirm here that CsA binds to CyP-D, inhibiting its PPIase activity and preventing its binding to the ANT. This would appear to be the mechanism by which CsA inhibits opening of the MPTP as we originally proposed (6). In contrast, SfA inhibits the PPIase activity of CyP-D (Fig. 1) and inhibits MPTP opening (Figs. 2–4) without preventing CyP-D binding to the ANT (Fig. 5). It seems probable that both CsA and SfA act to inhibit the MPTP via their binding to CyP-D because the concentration of each required to inhibit pore opening by >80% is almost identical (Fig. 3*a*). However, our data imply that SfA and CsA bind to CyP-D at different sites. In both cases the PPIase activity is inhibited, but only for CsA does binding of the inhibitor prevent binding of CyP-D to the ANT. Thus the SfA·CyP-D complex can still bind to the ANT but is unable to facilitate the conformational change, triggered by [Ca^{2+}], that induces pore opening. This allows a further conclusion to be drawn. Binding of CyP-D alone is not sufficient to promote pore opening; a subsequent conformational change, catalyzed by the PPIase activity of

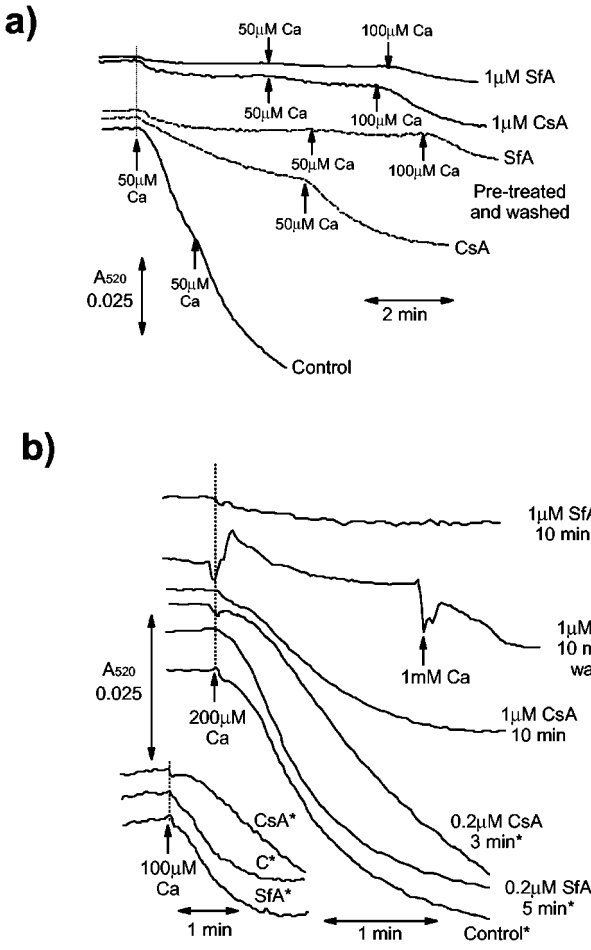

FIG. 6. **SfA and CsA inhibit the MPTP in the perfused heart.** In *panel a* isolated rat heart mitochondria (2 mg/ml in isolation buffer) were incubated in the presence or absence of 1 μM CsA or SfA for 10 min at 0 °C before sedimentation and two washes at 0.4 mg/ml. MPTP opening under de-energized conditions (1 mg protein/ml) was monitored (A_{520}) following addition of Ca^{2+} at the concentration shown. The *top two traces* represent control mitochondria to which 1 μM CsA and SfA have been added directly to the swelling assay, whereas the *middle traces* show mitochondria pretreated with the drugs and then washed. In *panel b* mitochondria were isolated from hearts perfused (Langendorff mode) for 30 min in the absence of drug and then with the concentration of CsA or SfA indicated for the times shown. Where indicated, extracellular SfA was washed from the heart prior to mitochondrial preparation by perfusing it for 30 s with SfA-free buffer (5 ml). Assay of MPTP under de-energized conditions was performed as described for *panel a* at a final protein concentration of 0.5 mg/ml. The *inset traces* were performed with the same mitochondria as the traces marked with an *asterisk* but with swelling initiated by addition of [Ca^{2+}] at 100 as opposed to 200 μM.

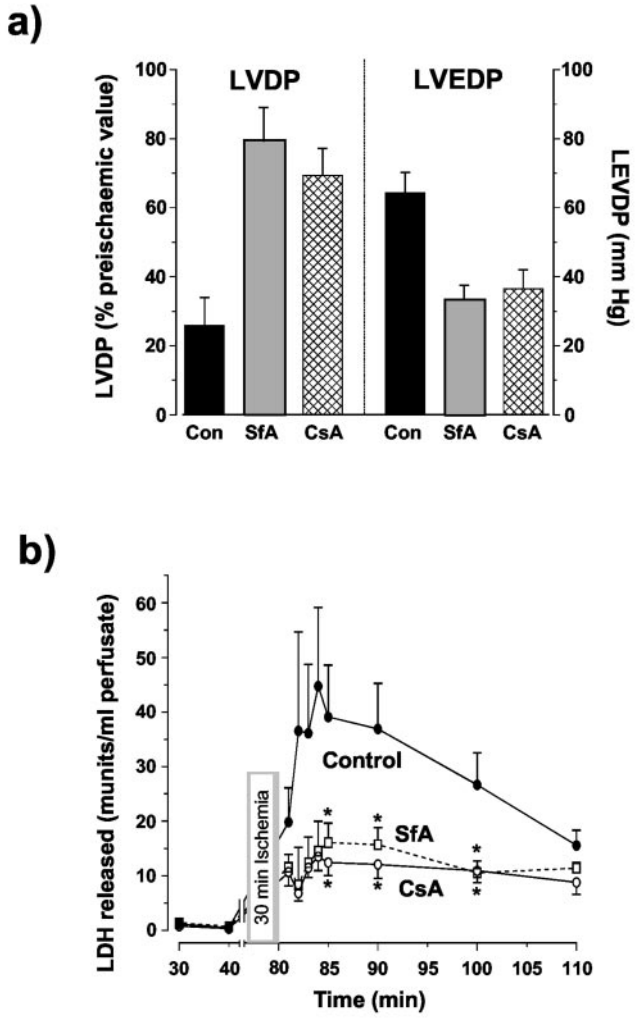

FIG. 7. **SfA and CsA protect the ischemic rat heart from reperfusion injury.** Rat hearts were perfused in the Langendorff mode, and the left ventricular pressure was monitored continuously as described under "Experimental Procedures." After 50 min, perfusion hearts were subject to 30 min of global isothermic ischemia followed by reperfusion. Where required, 0.2 μM CsA or 1 μM SfA was added 3 or 10 min, respectively, prior to ischemia. In *panel a*, greater functional recovery of the SfA- and CsA-treated hearts after a 30-min reperfusion is reflected in higher values for the LVDP and lower values for the LVEDP. Preischemic values for the LVDP and LVEDP were 76 ± 3.5 and 2.4 ± 0.3 mm Hg, respectively ($n = 9$) and were unaltered by either CsA or SfA. Values of the LVDP after reperfusion are presented as a percentage of the pre-ischemic value and were significantly greater ($p < 0.001$) with either SfA or CsA treatment, whereas values for the LVEDP were significantly lower ($p < 0.001$). In *panel b*, the release of lactate dehydrogenase (*LDH*) into the perfusate from the same hearts used in *panel a* was measured as an indicator of necrotic cell death (*, $p < 0.05$ for CsA- or SfA-treated hearts *versus* controls).

CyP-D, must then occur that is inhibited by SfA. CsA prevents the PPIase-dependent conformational change because it prevents CyP-D binding, whereas SfA still allows CyP-D binding but not the subsequent conformational change.

A further insight into the mechanism of the MPTP is provided by the sigmoidal nature of the concentration dependence of pore opening by SfA (Figs. 3 and 4). These data imply that SfA only inhibits pore opening once a significant proportion (about 50%) of the matrix CyP-D has bound SfA. This contrasts with CsA, where inhibition occurs directly in proportion to the amount bound to CyP-D (Fig. 3) (6, 7, 20). The most probable explanation of this observation is that the MPTP is a multisubunit complex, with the CyP-D-mediated conformational change required to open the pore involving co-operative interactions between the subunits. The simplest model, illustrated in Fig. 8, would be that the ANT, which exists as a dimer, must bind two CyP-D molecules to induce the conformational change required to prime the MPTP to open upon triggering with Ca^{2+}. Even if one of the two CyP-D molecules has SfA bound, the conformational change will occur, but if both of the bound CyP-D molecules are associated with SfA, the conformational change cannot occur. However, a CyP-D-independent conformational change of the ANT can also induce pore opening at high levels of [Ca^{2+}] (33), which may explain why pore opening can still be observed in the presence of the maximally inhibiting concentration of SfA when higher levels of [Ca^{2+}] are employed (Fig. 4*b*).

It should be noted that our data do not eliminate other models for the MPTP pore. Thus a membrane protein other

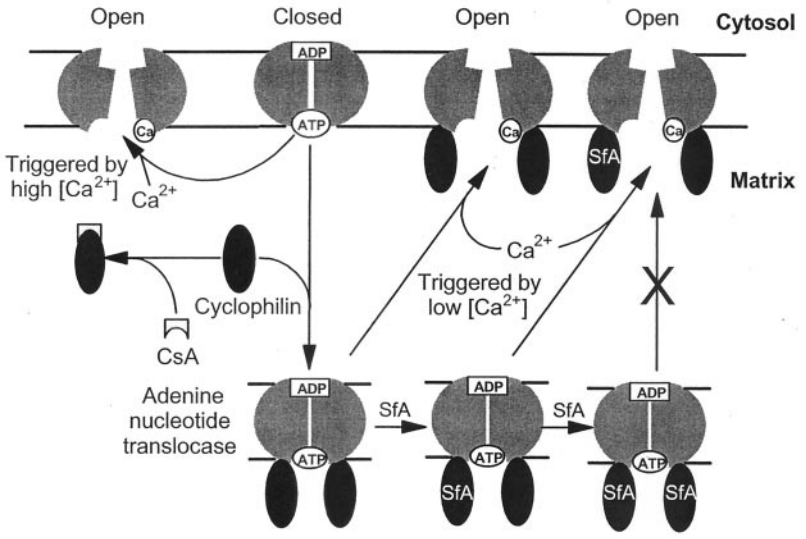

FIG. 8. **A scheme to account for the different modes of action of CsA and SfA on the MPTP.**

than the ANT may be the true target, or an additional target, of CyP-D binding that is responsible for MPTP formation. Indeed He and Lemasters (28) have suggested that many misfolded proteins may be capable of producing pores but are prevented from doing so by the binding of CyP-D and other chaperone proteins. This interaction may be disrupted by a calcium-mediated conformational change induced by the PPIase activity of CyP-D and blocked by CsA (28). Our data could be reconciled with such a model by substituting a denatured protein aggregate for the dimeric ANT shown in our scheme (Fig. 8). However, the well documented effects on the MPTP of adenine nucleotides and other ligands of the ANT, such as bongkrekic acid and carboxyatractyloside (18), are strongly suggestive that the ANT is the key membrane component that operates under most pathophysiological conditions. So too is the ability of the purified reconstituted ANT to form a nonspecific channel at high [Ca^{2+}] (33), which in the presence of CyP-D is opened at lower [Ca^{2+}] in a CsA-sensitive manner (2, 34).

SfA May Be Better Than CsA as an Inhibitor of MPTP-dependent Cell Death—To establish a role for the MPTP in apoptotic cell death, many workers have relied on the use of either CsA or bongkrekic acid as specific inhibitors of the MPTP. In reality, neither of these agents is specific. The use of bongkrekic acid is especially suspect, because its primary effect is to inhibit ATP/ADP transport across the mitochondrial inner membrane, a process that is fundamental to the metabolic and bioenergetic integrity of almost all cells. The use of CsA is open to criticism because when bound to CyP-A it inhibits the calcium-dependent protein phosphatase, calcineurin, which has many intracellular roles (8). This may, in part, explain why protection of hearts from reperfusion injury by CsA is critically dependent on its concentration, with optimal effects being observed at 0.2 μM (23, 29, 30). There are CsA analogues such as *N*-Me-Ala-6-cyclosporin A and *N*-Me-Val-4-cyclosporin A that do not inhibit calcineurin activity and yet still inhibit the PPIase activity of CyP-D, antagonize the opening of the MPTP and protecting cells from apoptotic and necrotic cell death (11–13). SfA also lacks the ability to inhibit calcineurin (14, 15) but has the additional advantage of binding more tightly to mitochondria than does CsA (Fig. 6). It thus represents an important new specific tool for exploring the role of the MPTP in cell death, which may have advantages over CsA analogues as a protective agent against reperfusion injury and other cellular stresses that cause cell death through MPTP opening. The immunosuppressant activity of SfA is exerted by blocking T cell proliferation in response to interleukin 2, through a mechanism involving an NFκB-mediated increase in the expression of the tumor suppressor genes *p53* and *p21*, with the latter binding to and inhibiting the appearance of cell cycle kinase activity cyclin E-Cdk2 (14, 16, 17). Whether this action of SfA on the cell cycle will have any detrimental effects when the drug is used to inhibit reperfusion injury is unknown, although the relatively short time scales involved in reperfusion after ischemia would suggest not.

In conclusion, SfA provides new clues to the molecular mechanism of the MPTP and represents an important additional tool for studying the role of the MPTP in both necrotic and apoptotic cell death. The lack of any effect of SfA on calcineurin activity may also make it an appropriate therapeutic agent for the protection of tissues from reperfusion injury. Two situations of particular importance in this regard are the treatment of stroke and coronary thrombosis, where reperfusion of the ischemic area following clot disruption may induce necrotic or apoptotic cell death. Finally, our data demonstrate that there are two distinct sites on CyP-D that may be targeted in the design of novel inhibitors of the MPTP that could be used therapeutically to protect from reperfusion injury.

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Sanglifehrin A Acts as a Potent Inhibitor of the Mitochondrial Permeability Transition and Reperfusion Injury of the Heart by Binding to Cyclophilin-D at a Different Site from Cyclosporin A

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