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CYCLOSPORIN A-SENSITIVE AND INSENSITIVE MECHANISMS PRODUCE THE PERMEABILITY TRANSITION IN MITOCHONDRIA

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Cyclosporin A is a potent inhibitor of the mitochondrial permeability transition, possibly by blocking an inner membrane pore through which solute movements occur [Broekemeier et al. (1989) J. Biol. Chem. 264, 7826-7830]. The inhibitory effect of cyclosporin, however, is transient. Trifluoperazine, at concentrations which inhibit the mitochondrial phospholipase A_2, also produces a transient inhibition. When both inhibitors are used together, the inhibitory effect is long lasting. These findings suggest that the transition can be caused by two overlapping and/or interactive mechanisms, one dependent on an inner membrane pore and the other on phospholipase A_2.

In the presence of Ca^{2+} and agents such as *t*-butylhydroperoxide, sulfhydryl reagents or inorganic phosphate (inducing agents), mitochondria undergo the so-called permeability transition which allows normally impermeant solutes to cross the inner membrane at significant rates (e.g., 1-3). Recent work indicates that the immunosuppressive peptide, cyclosporin A, is a potent inhibitor of the transition (4-7). The potency and apparent specificity of the cyclosporin A effect indicates that it interacts with a specific component which is required to produce the permeable state. It has been proposed that the component is a pore through which solute movements occur or a factor which regulates such a pore (4, 5).

Earlier work supported the notion that the permeability transition is caused by perturbation of a phospholipid deacylation-reacylation cycle involving inner membrane phospholipids. Ca^{2+} accumulation was thought to activate phospholipase A_2, the inducing agent was thought to inactivate reacylation, and the resulting accumulation of lysophospholipids and free fatty acids was thought to decrease the permeability barrier through actions on the membrane lipid phase (8). Considerable evidence supports this earlier proposal. Phospholipase A_2 reaction products accumulate as the transition occurs (1, 8, 9). Phospholipase A_2 inhibitors inhibit the transition (e.g., 10). Exogenous lysophospholipids cause the transition (11, 12). Authentic inhibitors of acyl-CoA:1-acyl-lysophospholipid acyltransferase cause the transition in a Ca^{2+}-dependent manner (13) and, furthermore, most other

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transition-inducing agents can be shown to inhibit acyltransferase activity by direct or indirect mechanisms (14).

If transition-dependent solute movements occur through a pore which is inhibited by cyclosporin A, then one is faced with integrating this model with the earlier data supporting perturbed phospholipid acyl group metabolism as a factor regulating the transition. Thus, we must consider the possibility that phospholipase A_2 reaction products are regulators of the pore. It also seems reasonable to ask if the cyclosporin-sensitive pore and the phospholipid deacylation-reacylation cycle are part of independent mechanisms which can create the same effect independently. In this report, we present data which bear on these possibilities.

MATERIALS AND METHODS

Rat liver mitochondria were prepared from male Sprague-Dawley rats by standard procedures (10). Incubations were conducted at 25°C and 1.0 mg protein/ml in media containing 10 mM succinate (Na^+) plus rotenone (0.5 nmol/mg protein), 3 mM Hepes (Na^+), pH = 7.4 plus sufficient mannitol-sucrose (3:1 ratio) to give a total osmotic strength of 300 mOsM. To determine the release of Ca^{2+} and Mg^{2+}, mitochondria were rapidly sedimented in a microcentrifuge and the cation concentration in supernatants was determined by atomic absorption measurements (15). When these data are expressed as a percent of total cation content, the fraction found outside the mitochondria immediately before the addition of the inducing agent was subtracted from the experimental values prior to calculation (15). Swelling was monitored by apparent absorbance measurements at 540 nm in the Aminco DW2a spectrophotometer. When the time courses of swelling and Mg^{2+} release were to be compared, samples were taken from a large incubation and transferred to a cuvette to determine apparent absorbance at 540 nm. A portion of the cuvette contents were then transferred to a microcentrifuge tube for centrifugation and the determination of Mg^{2+} release. This procedure avoids errors in time course comparisons which can arise from conducting the two measurements on separate incubations (15). All reagents were obtained from commercial suppliers except for cyclosporin A which was a generous gift from Dr. David L. Winter of Sandoz. Mannitol-sucrose was deionized before use.

RESULTS

Cyclosporin A inhibits large amplitude swelling associated with the permeability transition regardless of which agent is used to induce the phenomenon (4). I_{50} occurs at 25-50 nmol/mg protein with an apparent maximal effect obtained below 0.5 nmol/mg protein (4). Figure 1 shows the effect of the latter cyclosporin A level on release of Ca^{2+} and Mg^{2+} when the transition is induced by *t*-butylhydroperoxide. For both cations, it is apparent that the effect of cyclosporin A is transient. The inhibitor essentially eliminates cation release for approximately a 10-min period when compared to the control, but following that, complete release occurs. A similar effect is produced by the phospholipase A_2 inhibitor, trifluoperazine (10), except that it is somewhat less effective than cyclosporin A. More importantly, both compounds added together are much more effective than either one alone (Fig. 1).

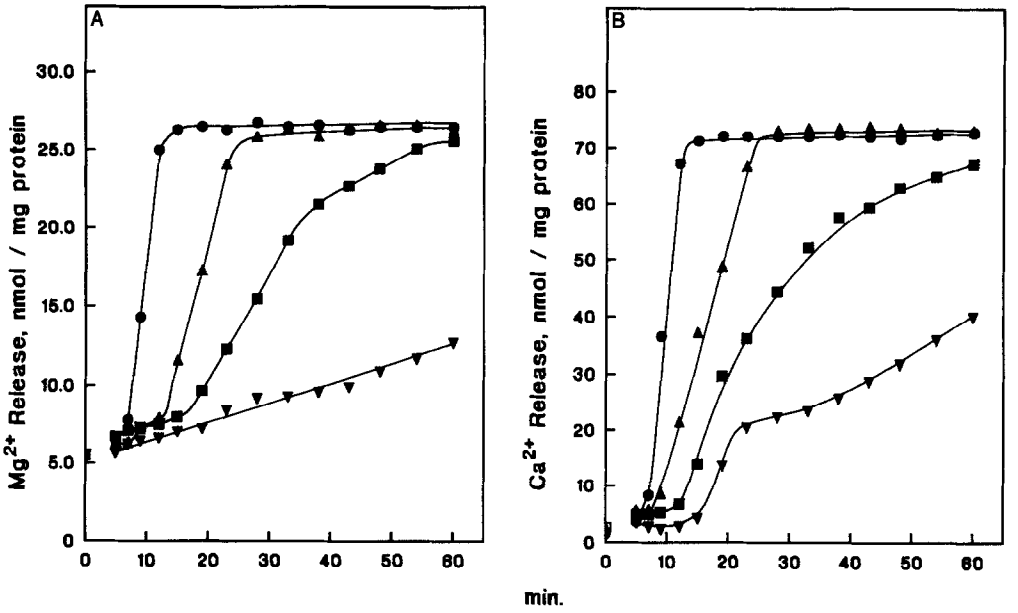

Figure 1. The effect of transition inhibitors on the release of cations induced by Ca^{2+} plus *t*-butylhydroperoxide. Mitochondria were incubated and the release of Ca^{2+} and Mg^{2+} were determined as described in Materials and Methods. $CaCl_2$ (70 nmol/mg protein) and *t*-butylhydroperoxide (200 μM) were added at 2 and 4 min, respectively, with experimental samples taken at the times shown. Panel A, Mg^{2+} release. Panel B, Ca^{2+} release. For both panels, ●, no further additions; ■, the medium contained trifluoperazine at 60 μM; ▲, the medium contained cyclosporin A at 0.5 nmol/mg protein (0.5 μM); ▼, the medium contained trifluoperazine and cyclosporin A at the levels described above. When utilized, inhibitors were present from the beginning of the incubation.

The data in Figure 1 suggest that there are two mechanisms for producing the permeability transition, one inhibited by cyclosporin A and other other by trifluoperazine. To test this possibility further, the concentration of the two inhibitors was varied. Figure 2A shows that the cyclosporin A effect is a function of the level employed up to approximately 1 nmol/mg protein. However, further increases in this value do not substantially improve the retention of Mg^{2+}. Similarly, the inhibitory effect of trifluoperazine is concentration-dependent over the range of 0-80 μM with the highest level still allowing complete Mg^{2+} release within 20 min following *t*-butylhydroperoxide addition (data not shown). When a near maximally effective level of cyclosporin A is employed, the further protection afforded by trifluoperazine is still dependent on the concentration of the second compound. These are the results expected for independent mechanisms.

In the present medium, swelling occurs because of mannitol-sucrose accumulation within the matrix space followed by H_2O uptake to maintain an osmotic balance. Plots of swelling response vs the Mg^{2+} release response, therefore, contain information on the relative membrane permeability to mannitol and sucrose vs the cation, after the transition has occurred (15).

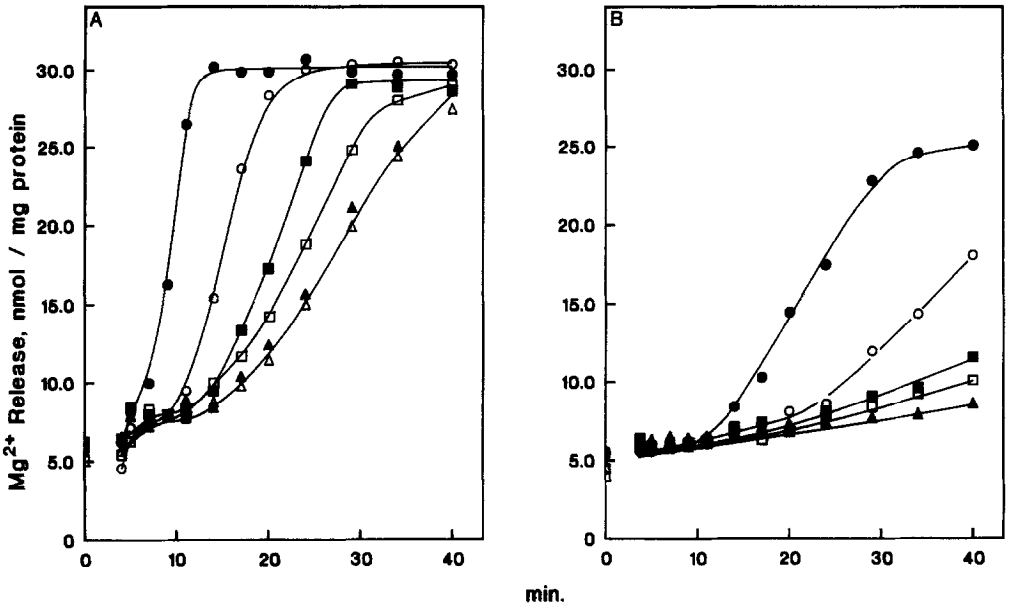

Figure 2. The effect of inhibitor concentration on the release of Mg^{2+} induced by Ca^{2+} plus t-butylhydroperoxide. Panel A, incubations were conducted as described in Materials and Methods and the legend to Figure 1A. No trifluoperazine was present and the level of cyclosporin A employed was varied as follows: ● , none; ○ , 0.075 nmol/mg protein; ■ , 0.25 nmol/mg protein; □ , 0.50 nmol/mg protein; ▲ , 1.0 nmol/mg protein; △ , 1.5 nmol/mg protein. Panel B, experiments are analogous to those in panel with cyclosporin A present at 0.5 nmol/mg protein. In addition, trifluoperazine was present as follows: ● , none; ○ , 20 μM; ■ , 40 μM; □ , 60 μM; ▲ , 80 μM.

As can be seen in Figure 3, Mg^{2+} release is slowed, relative to swelling, by the presence of trifluoperazine compared to what is seen in the absence of inhibitors or in the presence of cyclosporin A. This result would be expected if solutes traverse the membrane through more than one structure, if the structures differed somewhat in selectivity for Mg^{2+} vs the sugars, and if they were separately blocked by the two inhibitors.

DISCUSSION

It is clear that liver mitochondria contain phospholipase A_2 activity associated with the inner and outer membranes (16-18) and that activation of this enzyme is associated with the permeability transition (1, 8-10). It seems obvious that sufficient action by phospholipase A_2 will produce a marked reduction of the inner membrane permeability barrier. It was difficult to understand, therefore, how small amounts of cyclosporin A can inhibit the transition without inhibiting this enzyme. The results presented here suggest an answer to the apparent dilemma, namely that the transition can occur by at least two mechanisms which differ in inhibitor sensitivity. The cyclosporin A-sensitive mechanism may well represent the opening of an inner membrane pore or channel as has been proposed (4, 5). The trifluoperazine-sensitive

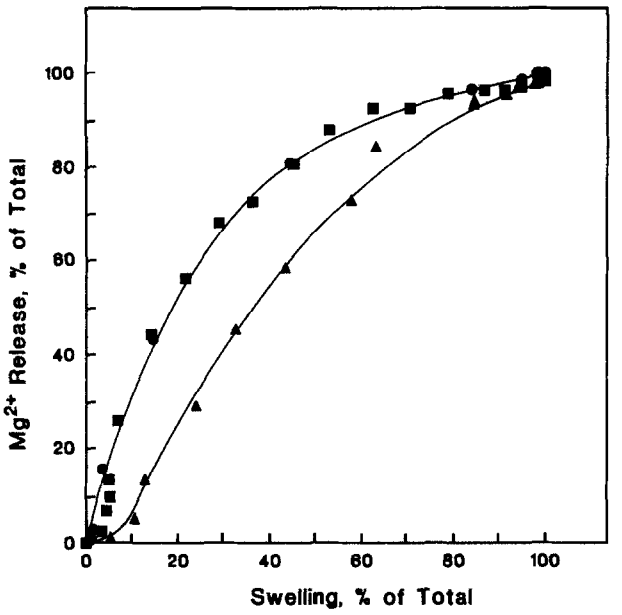

Figure 3. The effect of trifluoperazine and cyclosporin A on the relative time course of swelling and Mg^{2+} release induced by Ca^{2+} plus t-butylhydroperoxide. Incubations were conducted and swelling and Mg^{2+} release were determined as described in Materials and Methods and the legend to Figure 1. Data are expressed as percent of the matrix Mg^{2+} released as a function of the total swelling response (see Ref. 15 for a discussion of advantages to this form of presentation). The medium contained inhibitors as follows: ●, none; ■, cyclosporin A (0.5 nmol/mg protein); ▲, trifluoperazine (60 μM).

mechanism may well represent the action of accumulating phospholipase A_2 reaction products as has also been proposed (8, 10). To determine if this interpretation is correct, we are investigating the combined effect of trifluoperazine and cyclosporin A on the transition when induced by other agents and correlating these effects with the accumulation of phospholipase A_2 reaction products. The results will be presented elsewhere.

If the transition does indeed occur by two mechanisms, then they may well be overlapping and/or interactive. Overlap, with respect to time, is suggested by the fact that either inhibitor when used alone, reduces the rate of Mg^{2+} release compared to the control experiment. If one of the two mechanisms produced the transition substantially slower than the other, one would not expect the inhibitor of the slow mechanism to effect the rate when it is used alone. An effect of both inhibitors on the early time course of the transition would also be seen if the two mechanisms interact in such a way that inhibiting one slows down the other. This could occur, for example, if accumulating phospholipase A_2 reaction products made it easier to open the pore in addition to altering the permeability characteristics of the membrane lipid phase, or if opening the pore in some way increased activity of phospholipase A_2. In either case, interactive regulation of the two putative

mechanisms is an attractive possibility because it can explain why both mechanisms are activated by the same conditions (i.e., Ca^{2+} accumulation in the presence of an inducing agent).

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