Journal of Bioenergetics and Biomembranes, Vol. 24, No. 1, 1992

Research Paper

The Mitochondrial Megachannel is the Permeability Transition Pore[1]

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Single-channel electrophysiological recordings from rat liver mitoplast membranes showed that the 1.3-nS mitochondrial megachannel was activated by Ca^{++} and inhibited by Mg^{++}, Cyclosporin A, and ADP, probably acting at matrix-side sites. These agents are known to modulate the so-called mitochondrial permeability transition pore (Gunter, T. E., and Pfeiffer, D. R. (1990) *Am. J. Physiol.* **258**, C755–C786) in the same manner. Furthermore, the megachannel is unselective, and the minimum pore size calculated from its conductance is in agreement with independent estimates of the minimum size of the permeabilization pore. The results support the tentative identification of the megachannel with the pore believed to be involved in the permeabilization process.

KEY WORDS: Mitochondrial channels; selectivity; patch-clamp; permeability transition; calcium; magnesium; Cyclosporin A (rat liver mitochondria).

INTRODUCTION

The work of several groups has resulted in a fairly detailed catalogue of putative channels in the inner mitochondrial membrane. Perhaps the most studied of these "uniports" is the Ca^{++} carrier (Nicholls and Akermann, 1982; McCormack *et al.*, 1990; Gunter and Pfeiffer, 1990; Zoratti and Szabó, 1991). An unselective pathway for monovalent cations (Nicolli *et al.*, 1991), one which favors Na^+ and Li^+ over K^+ (Bernardi *et al.*, 1990) and an anion-selective channel (the IMAC) (Garlid and Beavis, 1986), can also be observed. Finally, exposure to high $[Ca^{++}]$, preferably in association with one of a variety of "inducing agents," causes the appearance of a giant, unselective "permeability transition pore" (PTP) (Gunter and Pfeiffer, 1990; Zoratti and Szabó, 1991), with a minimum diameter of 2.8 nm (Massari and Azzone, 1972), which has been found to be inhibited by Cyclosporin A (Fournier *et al.*, 1987; Crompton *et al.*, 1988; Broekemeier *et al.*, 1989; Novgorodov *et al.*, 1990). Among the other known effectors of the permeability transition, we mention here two inhibitors, namely Mg^{++} (Haworth and Hunter, 1979) and ADP (Harris *et al.*, 1979; Haworth and Hunter, 1980). The latter is particularly interesting: the effects of the adenine nucleotides and of known inhibitors of AdN exchange, most notably atractyloside and bongkrekate, have been interpreted to indicate that the permeability transition pore is actually formed by the adenine nucleotide exchanger (Davidson and Halestrap, 1990; Halestrap and Davidson, 1990; Novgorodov *et al.*, 1990). Crompton and Costi (1988) have presented evidence for cooperativity among the pores: within a single mitochondrion they appear to operate with a degree of synchrony. Recent results by Vercesi's group suggest that the pore might arise from the cross-linking via disulfide bridges of proteins of the inner mitochondrial membrane (Fagian *et al.*, 1990).

Our knowledge of the molecular properties of these channels ought to be greatly increased by the

[1] Abbreviations used: PT: permeability transition; PTP: permeability transition pore; MMC: mitochondrial megachannel; IMAC: inner membrane anion channel. P_A: permeability of ion A. CSP: Cyclosporin A.

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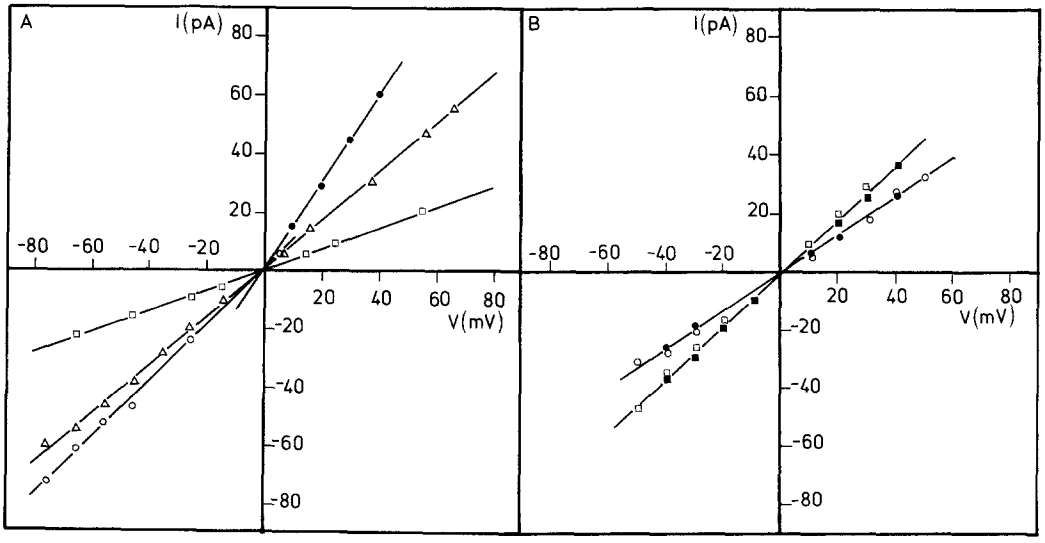

Fig. 1. Lack of selectivity of the mitochondrial megachannel. Examples of single-channel *I/V* curves. (A) K^+ vs. Cl^-. An experiment with 200 mM KCl in the patch pipette, and 50 mM KCl in the bath as asymmetrical ionic conditions. (●) *I/V* relationship of the highest conductance state (1.5 nS) under symmetrical (200 mM KCl) conditions. (○) *I/V* relationship of the highest conductance (0.97 nS) under asymmetrical ionic conditions. (△, □) *I/V* curves of two substate conductances (0.84 and 0.35 nS, respectively) under asymmetrical conditions. (Only two subconductance levels are shown for clarity). (B) Representative *I/V* curves from a patch in symmetrical 150 mM KCl (■, ●) and after substitution of the bath medium with 150 mM CsCl (□, ○). Conductances: 0.9 (□, ■) and 0.65 (○, ●) nS.

application of single-channel electrophysiological techniques. So far, these have led to the detection of a number of currents (Zoratti and Szabó, 1991) and to the unambiguous identification of two channels: a 107-pS slightly anion-selective channel (Sorgato *et al.*, 1987, 1989) and a 1.3-nS multi-conductance mitochondrial megachannel (MMC) (Kinnally *et al.*, 1989; Petronilli *et al.*, 1989). The latter appears to be formed by "units" exhibiting cooperative behavior, and might possess a binary structure, since it often exhibits conductance states close to half-maximal values. It has recently been found to be inhibited by Cyclosporin A (Szabó and Zoratti, 1991) and by the amphiphilic drugs amiodarone, propranolol, and quinine (Antonenko *et al.*, 1991).

Neither of these channels has yet been identified with certainty with one of the uniports listed above. Since the huge size of the MMC and its inhibition by CSP are clearly reminiscent of the permeability transition pore, we are working to establish or reject this identification. If it is indeed the PTP, the MMC ought *inter alia* to be unselective, to be induced by Ca^{++}, and to be inhibited by Mg^{++} and by ADP. We report here that all these conditions are fulfilled. We are currently extending this verification to other factors known to affect the PTP.

MATERIALS AND METHODS

The experimental procedures used to obtain single-channel electrophysiological recordings were as previously described (Petronilli *et al.*, 1989; Szabó and Zoratti, 1991). High-resistance seals were established on mitoplasts produced by subjecting rat liver mitochondria to an osmotic shock (30 mM Tris Cl), followed by washing with the desired medium. The mitoplasts became attached to the glass bottom of a chamber containing 0.8–1 ml of medium. Voltage gradients were applied in pulses of alternating sign, lasting 1 or 2 sec, separated by short intervals at zero potential. The filter cutoff frequency was 5 kHz and the data were recorded in digital form (sampling frequency 22 kHz). The bath was grounded via an agar bridge. Unless otherwise specified, experiments were conducted in symmetrical 150 mM KCl, 0.1 mM $CaCl_2$, and 20 mM Hepes/K^+, pH 7.2, at room temperature. Solutions of effectors (e.g., ADP, $CaCl_2$) were added in microliter amounts to the patch chamber, with a microsyringe. The chamber contents were mixed after additions by withdrawing and re-adding three times in succession approximately 300 μl aliquots of the chamber contents. This operation, lasting a few seconds, often introduced high 60-cycle noise into the

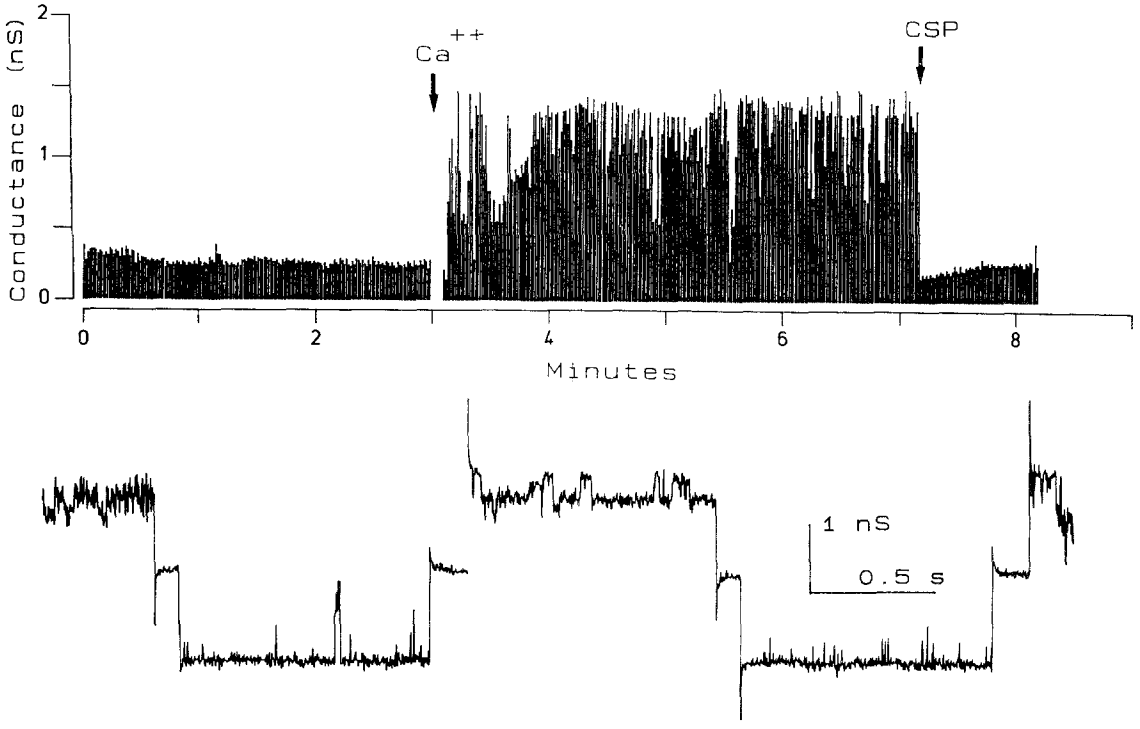

Fig. 2. Ca^{++} elicits MMC activity. (A) Total (leaks not subtracted) conductance of an excised patch, averaged over 0.82-sec intervals, vs. time. Arrows: additions of 5 μl of 0.1 M $CaCl_2$ and 10 μl of 2 mM Cyclosporin A, respectively. The chamber contained approximately 1 ml of medium. (B) Current records exemplifying channel activity after Ca^{++} addition. Digital sampling frequency: 5 kHz.

record. When this was the case, the record segment was not used (blank spaces in Figs. 2, 3, 5). Total patch conductance vs. time plots (Figs. 2, 3, 5) were obtained by averaging the conductance (I/V) values of the data points recorded during each voltage pulse, and plotting the average as a bar. Capacitance peaks were excluded.

For selectivity determinations, single-channel current-voltage relationships were determined from a membrane patch bathed in symmetrical KCl medium. The medium in the patch chamber was then substituted with the desired solution (KCl at a different concentration to determine P_K/P_{Cl}; M^+Cl^- or K^+X^- at the same concentration to determine P_K/P_M or P_{Cl}/P_X), and the I/V curve determined again. Two to five separate experiments were carried out for each ionic couple. Representative experiments are shown. Each point in the plots represents the average of a few individual measurements of events. The permeability ratios could be calculated from the reversal potential under asymmetric conditions by straightforward elaborations of the Goldman–Hodgkin–Katz equation (Hille, 1984). Cyclosporin A was kindly provided by Dr. Roemer of Sandoz Ltd.

RESULTS

A clear characteristic of the PTP is its lack of selectivity: the "permeabilized" mitochondria lose solutes ranging from KCl to nucleotides, and allow molecules as large as PEG 1500 to enter the matrix. The ability of the MMC to discriminate between cations and anions and among various cations or anions was checked by medium substitution experiments as described in the experimental section. In addition to the comparison between K^+ and Cl^-, experiments were carried out to compare the permeabilities of Na^+, Cs^+, and tetramethylammonium with that of K^+, and of I^-, acetate, and isobutyrate with that of Cl^-. As exemplified by Fig. 1, in all cases the I/V relationships of both maximal conductance states and substates intersected the axes at points within 5 mV of the origin. Given the conditions used,

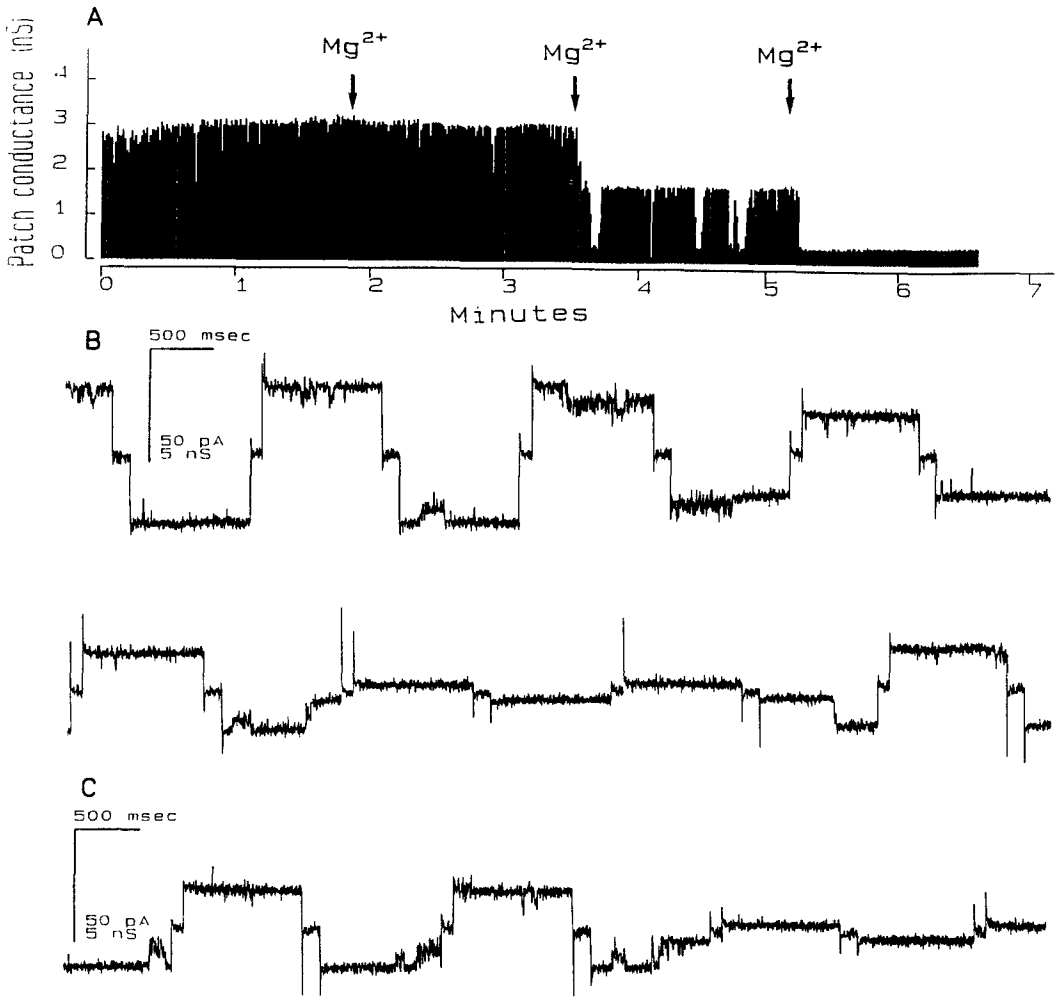

Fig. 3. Mg^{++} inhibits MMC activity. (A) Total conductance of an excised patch, averaged over 0.82-sec intervals, vs. time. When indicated, 2 μl aliquots of 0.1 M $MgCl_2$ were added. (B, C) Current records collected shortly after the second (B) and third (C) additions of $MgCl_2$. Digitizing frequency: 5 kHz. Voltage protocol: 1-sec, 10-mV pulses of alternating sign with separating intervals at 0 mV.

a selectivity higher than a factor of 2 can therefore be excluded for all the ionic couples tested. Attempts to study larger organic cations, such as tetraethylammonium, or anions, such as pivalate, $(CH_3)_3CCOO^-$, were unsuccessful because these compounds interfered with seal stability. The abundance of conductance levels complicated the experimental determination of I/V curves for the less-than-maximal conductances. However, in many cases reliable curves could be constructed, and also intersected the axes at the origin in all cases (Fig. 1). This behavior is consistent with the identification of the submaximal conductance levels as sublevels of the highest conductance, with the same lack of selectivity.

The relevance of isolation and medium ionic conditions for the activity exhibited by mitoplast membranes has been recently investigated by Kinnally *et al.* (1991). In our hands, about one-third of all patches (in symmetrical standard medium) were silent. Yet, in 46 out of 70 cases the addition of $CaCl_2$ (submillimolar range) to the patch chamber containing inactive patches resulted in the nearly instantaneous appearance of the typical current patterns due to the activity of one or more MMC's (Fig. 2). Presumably, in the 24 cases in which no activity was elicited, the patch contained no MMC's. In the case of active patches, addition of Ca^{++} often resulted in the recruitment of other, previously silent, channels as well as

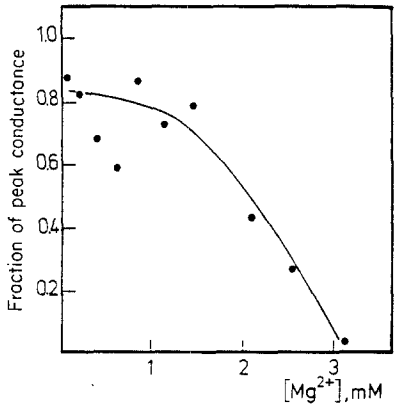

Fig. 4. A titration of MMC activity with Mg^{++}. $[Mg^{++}]$ was varied by adding microliter amounts of 0.1 M $MgCl_2$, followed by mixing. Mitoplast-attached patch. $[Ca^{++}] = 50\,\mu M$. Voltage protocol: 2-sec, 10-mV pulses of alternating sign. The patch contained three active MMC's. The leak-subtracted patch conductance was measured from conductance vs. time plots similar to those shown in Figs. 2, 3, and 5, averaging for a period of about 1 min after each addition. The averages plotted were divided by 3.9 nS (the expected maximal conductance for three MCC's).

in an increase of the open probability of the active channels (not shown). That the channels evoked were indeed MMC's was confirmed by the inhibitory effect of Cyclosporin A (Fig. 2), and by the characteristic voltage dependence: increasing positive potentials cause the MMC to function with increasing probability in lower-than-maximal conductance states, i.e., cause its progressive closure (not shown, see Petronilli *et al.*, 1989).

Mg^{++} inhibited MMC activity (in 15 out of 18 experiments). Figure 3 shows this in the case of an excised patch. With cell-attached mitoplasts inhibition also took place, but at somewhat higher concentrations, presumably reflecting the presence of the membrane permeation barrier. Figure 4 presents a titration of MMC activity obtained from a mitoplast-attached patch. In this experimental configuration the Mg^{++} concentrations needed were in the range found to inhibit the PT in suspensions of whole isolated mitochondria (Gunter and Pfeiffer, 1990). It must be mentioned that while these effects were qualitatively reproducible, quantitative differences from experiment to experiment were the rule. This might have been expected in view of the complexity and abundance of factors affecting the permeability transition pore (Gunter and Pfeiffer, 1990), which we made no attempt to control fully.

A further verification of the expected behavior was provided by experiments such as the one presented in Fig. 5: addition of submillimolar concentrations of ADP to membrane patches (either excised or in pipette-attached vesicles) exhibiting MMC activity brought about the immediate inhibition of the channels (in 18 experiments out of 19). The inhibition could not be readily reversed by increasing the Ca^{++} concentration: reversal occurred in only two out of eight attempts.

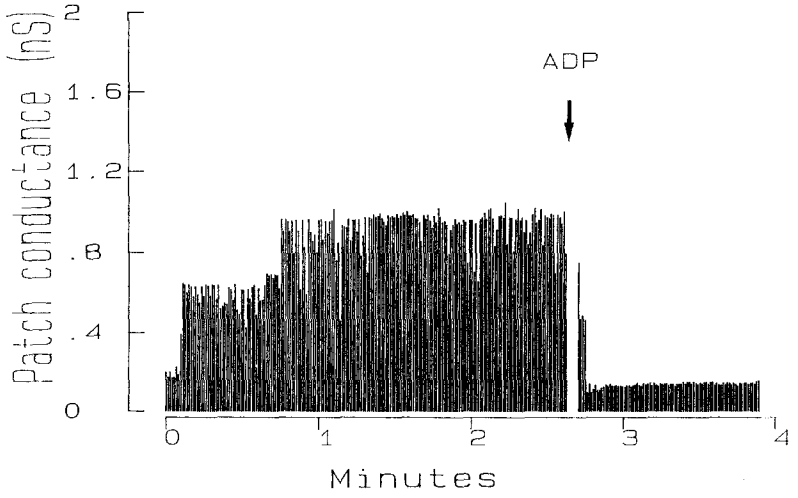

Fig. 5. ADP inhibits MMC activity. The patch conductance, averaged over 0.82-sec intervals, is plotted vs. time. Where indicated, 10 μl of 10 mM ADP/K^+ was added to the chamber (volume: 1 ml).

DISCUSSION

The modulation of MMC activity by Ca^{++}, Mg^{++}, and ADP, described above, closely matches the observations on the PTP (Gunter and Pfeiffer, 1990; Zoratti and Szabó, 1991). Because electrophysiological data were collected only from the membrane patch enclosed by the pipette rim, and since the pipette (cytoplasmic) side of this membrane is hardly accessible to reagents added into the bath, these compounds most likely act on the matrix side of the mitochondrial membrane. Together with the effect of CSP (Szabó and Zoratti, 1991), these results strongly suggest that the MMC can be identified with the pore believed to account for the permeability transition of mitochondria, which is modulated by the same agents in the same manner and in the same approximate concentration ranges. The various effectors act on all the different conductances exhibited by the MMC, including the major approximately 500, 350, and 250 pS ones, confirming that these are all manifestations of the same channel system. On the contrary, the behavior of the 107-pS channel is not affected by any of the conditions used. This "IMM" channel (Sorgato *et al.*, 1987) may therefore be presumed to be a different molecular species.

Support for the proposal comes from an estimate of the diameter of a 1.3-nS (150 mM KCl) pore treated as a medium-filled cylinder. Assuming a minimum length of 7 nm, the lower limit for the diameter may be calculated (Hille, 1984) at 2.7 nm, in agreement with the value provided by Massari and Azzone (1972). Such a large pore can hardly be expected to show any selectivity, except for extremely large solutes. Our results confirm that the pore does not select among ions either on the basis of charge or on the basis of size (within the limits tested).

The effects of Ca^{++} and ADP deserve special attention, in view of the possible involvement of the PTP/MMC in pathological phenomena in the aftermath of ischemia (Halestrap and Davidson, 1990; Crompton and Costi, 1988). Under those circumstances the cytoplasmic Ca^{++} levels are known to increase (Asimakis and Sordhal 1981; LaNoue *et al.*, 1981), and this would be expected to result in an increase in the mitochondrial matrix levels, with the consequent possible activation of the PTP/MMC. The matrix contains, however, millimolar levels of adenine nucleotides, which might be sufficient to ensure that the megachannels remain closed. Note that the mitoplasts used in our experiments had certainly lost their adenine nucleotides, because they had been subjected to an initial osmotic shock to eliminate the outer membrane (see Materials and Methods) and because of MMC operation.

The considerable body of available data concerning the PTP can now be exploited to predict and test MMC properties and behavior. The electrophysiological techniques should, on the other hand, allow progress in the clarification of the molecular mechanisms responsible for the onset of the PT, and of the properties of the pore itself.

ACKNOWLEDGMENTS

We thank Prof. G. F. Azzone for his interest, Drs. P. Bernardi and D. Pietrobon for discussions, and Dr. Roemer of Sandoz Ltd. for providing Cyclosporin A. This work was finance in part by a grant of the European Economic Community under the program "Science."

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