Modulation of the Mitochondrial Cyclosporin A-sensitive Permeability Transition Pore

1. EVIDENCE FOR TWO SEPARATE Me\(^{2+}\) BINDING SITES WITH OPPOSING EFFECTS ON THE PORE OPEN PROBABILITY*

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This paper reports an investigation on the regulation of the mitochondrial cyclosporin A-sensitive permeability transition pore (MTP). Energized, coupled rat liver mitochondria incubated in sucrose medium in the presence of phosphate maintain a high proton electrochemical gradient (\(\Delta$$\mu$$H\)) and a low permeability to solutes. Addition of a small (10–20 \(\mu\)M) Ca\(^{2+}\) pulse leads to a transient membrane depolarization. After Ca\(^{2+}\) accumulation, a high \(\Delta$$\mu$$H\) is recovered, and mitochondria remain coupled indefinitely. Yet, addition of fully uncoupling concentrations of carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone (FCCP) brings about MTP opening within seconds. This finding confirms that MTP opening is the consequence rather than the cause of membrane depolarization, and allowed us to study the operation of the MTP in a synchronized population of mitochondria, since pore opening can be triggered by the addition of uncoupler under a series of experimental conditions. We find that three regulatory sites can be defined: (i) an internal Me\(^{2+}\) binding site: when this site is occupied by Ca\(^{2+}\), the pore “open” probability increases, while other Me\(^{2+}\) ions (Sr\(^{2+}\), Mn\(^{2+}\)) have an inhibitory effect; (ii) an external Me\(^{2+}\) binding site: when this site is occupied by Me\(^{2+}\) ions, including Ca\(^{2+}\), the pore open probability decreases; (iii) an independent cyclosporin A binding site: when this site is occupied by cyclosporin A the pore open probability decreases. We show that at variance from the case of cyclosporin A, MTP inhibition by the phospholipase A\(_2\) inhibitors nupercaine and trifluoperazine is Ca\(^{2+}\)-competitive and is presumably related to interference by these drugs with Ca\(^{2+}\) binding to the internal regulatory site.

The maintenance of a low permeability to protons, anions, and cations generally is an integral part of mitochondrial energy conservation, as stated in Mitchell’s original formulation of the chemiosmotic hypothesis (1, 2). It is because of the insulating properties of the coupling membrane that H\(^+\) pumping at the energy-conserving sites of the respiratory chain can be stored in the form of a proton electrochemical gradient (\(\Delta$$\mu$$H\)), which in turn can be used for ATP synthesis (1, 2). In keeping with the needs for oxidative phosphorylation, energy drain linked to futile cation cycling through coupling of electrophoretic cation uptake (3) and electroneutral cation efflux (4) is kept to a minimum by restricting the rate of cation entry (5). It is therefore not surprising that conditions leading to unspecific increase of mitochondrial permeability are widely considered as a threat to mitochondrial integrity.

A well known case of mitochondrial permeability increase is the “permeability transition,” a condition that can be easily observed after Ca\(^{2+}\) accumulation in the presence of a surprisingly wide variety of inducing agents with no obvious functional or structural features in common (6). Typically, after a lag phase during which the membrane permeability remains normal, Ca\(^{2+}\) uptake is followed by a spontaneous permeability increase. This is due to opening of unselective channels with a minimum diameter of 2.8 nm (7) allowing equilibration of solutes with molecular masses up to 1200 daltons. Since the transition is inhibited with high affinity by the cyclic immunosuppressive endecapeptide cyclosporin A (8–11), it is now generally hypothesized that the transition is mediated by a proteinaceous pore (MTP) reversibly modulated by Ca\(^{2+}\) ions, as first suggested by Hunter and Haworth (12–14). This hypothesis is strongly supported by our demonstration that the MTP coincides with the mitochondrial megachannel (5, 15–17) previously identified by patch-clamp studies of rat liver mitoplasts (18). It is extremely likely, however, that multiple regulatory steps are involved in the modulation of pore activity, since the permeability transition can be inhibited with lower affinity by a wide variety of agents ranging from Me\(^{2+}\) ions to local anesthetics to scavengers of oxygen radicals and many others with no common structural features (see Ref. 6 for a review).

The function of the MTP is not known, but recent work from our laboratory indicates that the pore may be regulated, since it is modulated by the components of the \(\Delta$$\mu$$H\): the MTP is closed when matrix pH is lower than 7.0 (16) and/or when the membrane potential is high (19), while it opens upon membrane depolarization (19). In this paper, we have exploited these features to study the kinetics of MTP induction in a synchronized population of mitochondria. We find that mitochondria that have accumulated a small Ca\(^{2+}\) pulse...
in the presence of phosphate do not undergo spontaneous MTP opening, yet addition of uncoupler can trigger the transition within seconds. The amount of Ca" that had been accumulated essentially determines the rate of the ensuing swelling process, while externally added Ca" in the presence of ruthenium red has an inhibitory effect on the MTP. We show that three regulatory sites can be defined: (i) an internal Me" binding site: when this site is occupied by Ca", the pore "open" probability increases, while other Me" ions (Sr"", Mn"') have an inhibitory effect; (ii) an external Me" binding site: when this site is occupied by Me" ions, including Ca", the pore open probability decreases; (iii) an independent cyclosporin A binding site: when this site is occupied by cyclosporin A the pore open probability decreases. At variance from the case of cyclosporin A, MTP inhibition by the phospholipase A2 inhibitors nupercaine and trifluoperazine is Ca'-competitive, and is presumably related to interference of these drugs with Ca" binding to the internal regulatory site.

MATERIALS AND METHODS

Preparation of rat liver mitochondria and measurements of oxygen consumption were performed as described previously (20). Mitochondrial volume changes were determined either from absorbance changes at 540 nm as described in Ref. 16, or from the changes of 90° light scattering at 545 nm (22) with a Perkin-Elmer 650-40 Spectrophotometer equipped with magnetic stirring and thermostatic control. Measurements of matrix pH and membrane potential were carried out exactly as described previously (16, 19). Ruthenium red was purified according to Luft (21), and solutions were prepared daily. Ruthenium red concentrations were determined spectrophotometrically based on an extinction coefficient of 68 × 108 M"1 cm"1 at 533 nm. All chemicals were of the highest purity commercially available, while cyclosporin A was a generous gift of Sandoz Pharma AG (Basel).

Routine atomic absorption determinations revealed that our standard isotonic sucrose media, specified in the figure legends, contained 5 to 7 μM adventitious Ca". To optimize reproducibility we have routinely included 20 μM EGTA-Tris in all incubations. Whenever ruthenium red was used, the glass cuvettes were washed with incubation medium containing about 5 mg × ml"1 mitochondrial protein. The details of the experimental protocols are given in the figure legends.

RESULTS

In the experiments depicted in Fig. 1, respiring mitochondria were incubated in an isotonic sucrose medium containing 1 mM P, and 20 μM EGTA. After mitochondrial volume equilibration, followed here as the absorbance at 540 nm, a Ca" pulse of increasing size was added. After a small absorbance increase, presumably due to calcium phosphate matrix complexation, mitochondria maintained a stable volume. If, however, FCCP was added after Ca" uptake a fast process of absorbance decrease ensued, revealing an increased permeability to sucrose leading to matrix swelling. As clearly shown in Fig. 1, the rate of swelling induced by FCCP increased with the size of the Ca" load. Parallel measurements of Ca" uptake and membrane potential were performed. As expected, all Ca" was taken up and retained until FCCP was added, while mitochondria recovered a high membrane potential after transient depolarization linked to Ca" uptake (not shown). When the Ca" load was in excess of 30 μM a tendency to spontaneous depolarization was observed at longer incubation times (not shown). From the traces of Fig. 1, however, it is clear that no spontaneous permeability increase to sucrose took place within the time frame of these experiments. As we will show later (see Figs. 7 and 8), the uncoupler-induced permeability increase was fully sensitive to cyclosporin A, and is therefore mediated by the MTP.

The experiments of Fig. 2 were designed to assess the effect of external Ca" on the kinetics of MTP opening induced by

FIG. 1. Effect of the Ca" load on uncoupler-induced permeability transition. The incubation medium contained 0.2 M sucrose, 10 mM Tris-Mops, pH 7.4, 5 mM succinate Tris, 1 mM P, Tris, 20 μM EGTA-Tris, 2 μM rotenone, 1 μg × ml"1 oligomycin. Final volume was 2 ml, 25 °C. The experiments were started by the addition of 1 mg of mitochondria (not shown). After exactly 1 min, the indicated Ca" concentrations were added (first arrow), followed after 2 further minutes by FCCP (second arrow).

FIG. 2. Effect of external Ca" and EGTA on uncoupler-induced permeability transition. Experimental conditions were exactly as described in the legend to Fig. 1. One minute after the mitochondria, 50 μM Ca" was added (first arrow), followed where indicated (arrows) by 0.1 μM ruthenium red (RR), 100 μM EGTA-Tris, 100 μM CaCl2, and 0.2 μM FCCP. FCCP. After accumulation of a Ca" pulse that did not cause pore opening per se, ruthenium red was added to prevent the uptake of externally added Ca". Upon addition of FCCP the expected opening of the MTP took place, and mitochondria underwent a fast swelling process (trace a). If 100 μM EGTA was added after ruthenium red to chelate any external Ca", the subsequent rate of swelling following addition of FCCP was unaffected (trace b). Quite unexpectedly, if 100 μM Ca" was added instead of EGTA the rate of FCCP-induced swelling was significantly reduced (trace c).
This point is investigated further in Fig. 3, which reports the concentration dependence of MTP inhibition by external Ca$^2+$ as obtained from experiments analogous to the ones depicted in Fig. 2. Following the additions of ruthenium red and EGTA for each Ca$^2+$ load. See Fig. 2 for the actual absorbance traces after further 10 minutes. Following Ca$^2+$ loads of two different sizes, ruthenium red was added, followed by EGTA and increasing Mg$^{2+}$ concentrations before FCCP, in a protocol identical to that described above for Ca$^2+$. Fig. 4 shows that the same pattern of inhibition was obtained, with an $I_{50}$ of about 0.3 mM Mg$^{2+}$. Also in the case of Mg$^{2+}$, the $I_{50}$ was independent of the Ca$^{2+}$ load (inset). An inhibitory effect could also be observed with external Mn$^{2+}$ and Sr$^{2+}$ (not shown), while we have confirmed that accumulation of Sr$^{2+}$ and Mn$^{2+}$ has an inhibitory effect on MTP opening (24).

Thus, the data presented so far indicate that the MTP open-closed probability is modulated by two Me$^{2+}$ sites that can be distinguished experimentally: (i) an internal site: Ca$^{2+}$ binding to this site brings about an increased open pore probability, while binding of other Me$^{2+}$ ions (Sr$^{2+}$, Mn$^{2+}$) has the opposite effect; (ii) an external site: binding of Me$^{2+}$ ions, including Ca$^{2+}$, to the external site brings about an increased "closed" probability.

Recent data from our laboratory have indicated that a key factor affecting MTP activity is the transmembrane electrical potential (19). Since pore opening can only occur after membrane depolarization (19), this observation raises the possibility that many agents known to prevent spontaneous MTP opening after Ca$^{2+}$ overload may in fact prevent membrane depolarization rather than act as true pore blockers. The next question we have addressed is therefore whether three agents known to prevent spontaneous pore opening, the phospholipase A$_2$ inhibitors nupercaine and trifluoperazine and the immunosuppressant cyclosporin A, are indeed pore inhibitors in mitochondria depolarized with uncoupler. Figs. 5 and 6 report results from experiments where MTP opening was achieved by addition of FCCP to mitochondria that had accumulated Ca$^{2+}$ loads of different sizes in the presence of the indicated concentrations of nupercaine (Fig. 5) or trifluoperazine (Fig. 6). With both drugs the rate of FCCP-induced swelling decreased in a dose-dependent fash-
ion. Interestingly, with these inhibitors the I_50 was markedly dependent on the amount of Ca^{2+} that had been taken up, in that higher inhibitor concentrations were required for a comparable degree of inhibition when the Ca^{2+} load was higher (Figs. 5 and 6, insets). It must be mentioned that the inhibitory titer of nupercaine and trifluoperazine did not change if the drugs were added immediately prior to FCCP rather than at the beginning of the incubations (not shown). This observation suggests that nupercaine and trifluoperazine may inhibit pore opening by a direct effect on the pore, possibly by modifying Ca^{2+} binding at the internal activatory site, rather than by virtue of their inhibitory effect on phospholipase A_2 activity. It can be noted that higher concentrations of nupercaine or trifluoperazine could not be used because they caused cyclosporin A-insensitive swelling per se (not shown). This finding may partly explain why pore inhibition by these agents was not complete (Figs. 5 and 6).

In the experiments of Fig. 7, panel A, a 40 μM Ca^{2+} pulse was added to energized mitochondria incubated in the presence of 1 mM Pr and 20 μM EGTA in a sucrose-based medium. After the initial contraction accompanying Ca^{2+} uptake, a slow process of absorbance decrease ensued (panel A, trace a), which would eventually lead to the permeability transition at longer incubation times (not shown). Addition of FCCP prior to spontaneous MTP opening caused the expected rapid phase of matrix swelling (panel A, trace a). If 17 nM cyclosporin A was present the spontaneous slow phase of absorbance decrease was prevented, but FCCP-induced swelling was inhibited only partially (panel A, trace b), while complete inhibition of uncoupler-induced MTP opening required a cyclosporin A concentration of 68 nM (panel A, trace c). In the experiments of panel B, the size of the Ca^{2+} pulse was 80 μM. In this case, the transient phase of matrix contraction was followed by spontaneous opening of the MTP with matrix swelling within a few minutes of the addition of Ca^{2+} (panel B, trace a). If 17 nM cyclosporin A was present the spontaneous transition was inhibited, but a slow phase of absorbance decrease followed the transient contraction, while addition of FCCP was followed by a rapid swelling phase (panel B, trace b) which was as fast as the spontaneous swelling observed in the absence of cyclosporin A (compare with trace a in the same panel). Complete inhibition of the slow initial phase of absorbance decrease was obtained at 85 nM cyclosporin A, but some MTP opening could still be observed upon addition of FCCP (panel B, trace c). Only at 170 nM cyclosporin A was FCCP-induced swelling inhibited completely (panel B, trace d). The data in this experiment clearly indicate that cyclosporin A is a high affinity inhibitor of the permeability transition, but that the inhibitor titer required for full inhibition may change significantly depending on experimental variables such as the Ca^{2+} load and the membrane potential.

This point was investigated further in the experiments reported in Fig. 8. Mitochondria were allowed to accumulate increasing Ca^{2+} loads in the presence of the concentrations of cyclosporin A indicated on the abscissa. Opening of the MTP was then triggered by the addition of uncoupler, and the kinetics of pore opening was measured as the rate of 90° light scattering decrease at 545 nm (12). Fig. 8 shows that a dose-dependent inhibition could be observed. Interestingly, the I_50 for cyclosporin A inhibition was independent of the Ca^{2+} load (inset), suggesting that Ca^{2+} and cyclosporin A affect the pore open-closed probability by independent mechanisms. As we
shall discuss later, this observation may have practical implications.

**DISCUSSION**

**General Features of MTP Opening by Depolarization**—The present paper describes a reliable method to induce opening of the MTP in a population of mitochondria by simultaneous membrane de-energization. This method rests on our recent demonstration that pore opening can be triggered by membrane depolarization (19) provided that matrix pH does not drop below 7.0 (16, 19). In the present experimental protocols we have therefore (i) included P, in the incubation media, thus preventing major changes of pH, both during Ca\(^{2+}\) uptake end after membrane de-energization (22); (ii) used very low Ca\(^{2+}\) loads that do not cause pore opening per se, and allow recovery of the membrane potential after Ca\(^{2+}\) uptake; and (iii) triggered the transition by simultaneous depolarization of mitochondria with uncoupler after Ca\(^{2+}\) uptake had been completed.

With this method the pore will be manifest simultaneously throughout the mitochondrial population, but swelling data cannot show whether or not all pores in all mitochondria are opening simultaneously and therefore it still might be that some mitochondria complete the transition and swelling process faster than others. Despite this limitation, which should be borne in mind, several useful indications emerge from these experiments.

First, mitochondria that have accumulated a limited Ca\(^{2+}\) load have a functionally competent MTP. Indeed, upon addition of FCCP the MTP opens within seconds, and the transition then proceeds at a remarkably linear rate and is completed in a few minutes at most (Figs. 1 and 2). It must be stressed that since the MTP remains silent at high membrane potentials (19), its presence would go unnoticed as long as energy coupling is maintained. Furthermore, unless mitochondrial volume is being monitored, one may not suspect the presence of an open pore even after addition of uncoupler, since the membrane energy parameters (e.g. rate of respiration and \(\Delta\mu_H\)) will indicate a maximum of energy drain irrespective of whether H\(^+\) backflow is occurring via the MTP or the uncoupler. Thus, the role of MTP in mitochondrial physiology may have been largely underestimated, since most incubation media contain adventitious Ca\(^{2+}\) well within the range necessary for MTP induction, while P, is obviously required in studies of oxidative phosphorylation.

A second point of interest is represented by the fact that in our experiments pore opening is triggered by simultaneous de-energization of the mitochondria. A definite advantage of this protocol is that Ca\(^{2+}\) leaving the matrix after MTP opening cannot be taken up again. This is at variance from the case of the spontaneous MTP opening of Ca\(^{2+}\) overload where complex cooperativity phenomena occur, due to Ca\(^{2+}\) redistribution among mitochondrial subpopulations of different stability as the transition proceeds (6). An additional point emerging from these experiments should be appreciated. Since the rate of FCCP-induced swelling becomes faster as internal Ca\(^{2+}\) is increased (e.g. Fig. 1), while external Ca\(^{2+}\) has an inhibitory effect (Figs. 2 and 3); and since excess EGTA does not affect the rate of FCCP-induced swelling (Fig. 2), then pore opening under these conditions must precede Ca\(^{2+}\) efflux (see also Ref. 22).

A third consideration concerns the kinetics of pore opening. Spontaneous MTP opening in Ca\(^{2+}\) overload is characterized by a lag phase of variable length during which the membrane permeability remains normal, followed by the actual permeabilization phase. The lag phase is usually longer than strictly required for the process of Ca\(^{2+}\) uptake, and has therefore been considered to reflect a process of pore "assembly" whose biochemical basis remains obscure (19). In striking contrast with the case of Ca\(^{2+}\) overload, FCCP-induced pore opening does not require a lag phase of minutes and MTP opening occurs within seconds of the addition of uncoupler (e.g. Figs. 1 and 2). This finding strongly suggests that the lag phase of spontaneous pore opening reflects the time required for Ca\(^{2+}\)-dependent mitochondrial depolarization below a critical level (19) rather than a process of "pore assembly." On the other hand, a short time interval elapses between addition of FCCP and pore opening. This suggests that the coupling between membrane potential and the pore open-closed state may be indirect in nature, e.g. mediated by binding-unbinding of a voltage sensor. Work in progress in our laboratory indicates that ADP, which is known to inhibit MTP opening (12, 26, 27), is a likely candidate for this role. ADP may have a fundamental regulatory role on MTP operation is also suggested by the recent finding that ADP potentiates the inhibitory effect of cyclosporin A (28).

**Membrane Potential: Inhibitors of the MTP**—Data in this and in a previous paper (19) clearly show that MTP opening follows membrane depolarization. The possibility therefore exists that several inducers in fact be agents able to cause membrane depolarization, as is the case for the SH group bifunctional reagent phenylarsine oxide (19). Conversely, it appears quite possible that several low-potency inhibitors of the transition may be exerting their "protective" effects indirectly by preventing, or delaying, membrane depolarization.

It is because of these considerations that we tested whether the phospholipase A\(_2\) inhibitors nupercaine and trifluoperazine, and the immunosuppressant cyclosporin A, known to inhibit spontaneous MTP opening in Ca\(^{2+}\) overload (8-11, 28), would inhibit FCCP-induced pore opening as well. The data of Figs. 5 and 6 indicate that this is the case, and that these drugs are bona fide MTP inhibitors. Based on the apparent \(I_0\) (Figs. 5, 6, and 8) it appears that nupercaine and trifluoperazine are inhibitors of moderate affinity, while cyclosporin A is a high affinity inhibitor. It is important to recall that the same inhibitory titer could be obtained by adding nupercaine or trifluoperazine before Ca\(^{2+}\) or immediately prior to FCCP, strongly suggesting that the effect of these drugs on the pore may be independent of their inhibition of Ca\(^{2+}\)-activated phospholipases. Since the inhibitory titer increases as the Ca\(^{2+}\) load is increased (Figs. 5 and 6), and since these inhibitors are known to compete with Ca\(^{2+}\) for binding to mitochondrial membrane sites (30), we suggest that nupercaine and trifluoperazine exert their effects by perturbing Ca\(^{2+}\) binding to the internal Me\(^{2+}\) site. On the other hand, it appears that cyclosporin A exerts its inhibitory effect independently of Ca\(^{2+}\). This is at variance from what we found in the case of spontaneous MTP opening induced by phenylarsine oxide in de-energized mitochondria, where Ca\(^{2+}\) and cyclosporin A appeared to compete for the same site(s) (16). In that study, however, we were not yet aware of the role of the external Me\(^{2+}\) site (this paper) and of the membrane potential (19) in the regulation of the pore open-closed probability. It seems conceivable that a competitive inhibition between Ca\(^{2+}\) and cyclosporin A in de-energized, nonsynchronized mitochondria may have resulted from Ca\(^{2+}\)-dependent modifications of regulatory parameters other than the cyclosporin A site (internal pH via alteration of Donnan

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2 We acknowledge one of the reviewers for writing this part of the paragraph. We couldn't have made this point more clearly.

3 V. Petronilli and P. Bernardi, manuscript in preparation.
potential, Me$^{2+}$ occupancy of the internal and external sites).

**Me$^{2+}$ Ions and Modulation of the MTP**—Data in this paper show that two separate Me$^{2+}$ binding sites can be distinguished experimentally: (i) an internal Me$^{2+}$ binding site: when this site is occupied by Ca$^{2+}$ the pore open probability increases, as shown by the increased rate of FCCP-induced swelling as the Ca$^{2+}$ load is increased (Fig. 1); on the other hand, when this site is occupied by other Me$^{2+}$ ions that are transported by the Ca$^{2+}$ unipporter, and therefore are accumulated in the matrix (Mn$^{2+}$, Sr$^{2+}$), the pore open probability decreases (24); (ii) an external Me$^{2+}$ binding site: when this site is occupied by any Me$^{2+}$ ion, including Ca$^{2+}$, the pore open probability decreases, as shown by the decreased rate of FCCP-induced swelling. It appears that MTP modulation at the two sites occurs via independent mechanisms, since the inhibitory titer of external Me$^{2+}$ ions does not change if the Ca$^{2+}$ load is varied in a range that effectively increases the pore open probability (Figs. 3 and 4).

While the existence of an external binding site for Me$^{2+}$ whose occupancy results in pore inhibition has been generally inferred from Mg$^{2+}$ inhibition of the spontaneous MTP opening in Ca$^{2+}$ overload (6), two novel features of the present work should be appreciated. First, our finding that Mg$^{2+}$ inhibits pore opening induced by FCCP rules out the possibility that the inhibition by Mg$^{2+}$ is mediated by effects on the membrane potential. Second, our results clearly indicate that Ca$^{2+}$ itself may be a pore inhibitor when present on the cytosolic side of the membrane, and that Ca$^{2+}$ redistribution between the external inhibitory site and the internal stimulatory site may play a role in the regulation of the pore open-closed probability (22).

**Conclusions**—Although the physiological role of the MTP remains unclear, recent work from our laboratory clearly indicates that the pore is regulated, and the regulatory parameters are now beginning to emerge: (i) matrix pH: protonation of an inner site appears to lock the pore in the closed conformation (16); at pH of approximately 6.8 or less the pore will indeed not open irrespective of membrane depolarization (19); (ii) membrane potential: when pH is maintained at or above about 7.0 and the internal Me$^{2+}$ site is occupied by Ca$^{2+}$, the pore will favor the closed conformation at high membrane potential, and the open conformation upon depolarization (Ref. 19 and the present paper); (iii) Me$^{2+}$ ions: binding of Me$^{2+}$ ions to the internal and external sites independently modulates the pore open-closed probability (present paper). The complexity of MTP regulation suggests that whether the pore is open or closed for an individual mitochondrion at any given time depends on the interaction of many parameters. Since MTP opening is reversible (e.g. by removal of Ca$^{2+}$ from the internal site, Refs. 13 and 31) it appears that our findings may help in defining the bioenergetic basis for fine regulation of pore operation in vivo. How pore modulation by these effectors can at least explain the requirements for pore induction by a variety of agents in vitro is analyzed in more detail in the companion paper (22).

**REFERENCES**