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Modulation of the Mitochondrial Megachannel by Divalent Cations and Protons*

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In patch-clamp experiments on rat liver mitoplasts, the 1.3 nanosiemens (in 150 mM KCl) mitochondrial megachannel was activated by Ca^{2+} and competitively inhibited by Mg^{2+}, Mn^{2+}, Ba^{2+}, and Sr^{2+}. Cyclosporin A, which inhibits the megachannel, also showed a competitive behavior *versus* Ca^{2+}. The pore is regulated by pH in the physiological range; lower pH values cause its closure in a Ca^{2+}-reversibll manner. The modulating sites involved in these effects are located on the matrix side of the membrane. As illustrated in the companion paper (Bernardi, P., Vassanelli, S., Veronese, P., Colonna, R., Szabó, I., and Zoratti, M. (1992) *J. Biol. Chem.* 267, 2934-2939), the calcium-induced permeability transition of mitochondria is affected by these various agents in a similar manner. The results support the identification of the megachannel with the pore believed to be involved in the permeabilization process. The kinetic characteristics of the single channel events support the idea that the megachannel is composed of cooperating subunits.

The inner mitochondrial membrane has long been considered to be an almost impenetrable barrier for solutes that are not endowed with specific transport systems (1). For example, the proton conductance of isolated mitochondria, incubated in the resting energized state, has been estimated at 0.1-0.4 ng ion × min^{-1} × mg $protein^{-1}$ × mV^{-1} (2-5), *i.e.* about 25-100 ions × $s^{-1}/\mu m^{-2}$ of inner membrane (taking the inner membrane surface as 4 × 10^{10} μm^2 mg $protein^{-1}$ (6)). The permeability barrier breaks down if the mitochondria accumulate more than 30-50 nmol × mg $protein^{-1}$ Ca^{2+} in the presence of inducers (see below) (7-9). The transmembrane potential collapses, the mitochondria release the accumulated Ca^{2+} as well as K^+, Mg^{2+}, and other matrix components, and the matrix becomes accessible to external solutes such as sucrose. The phenomenon may be associated with the modification of membrane thiol groups (10, 11), and it may involve the formation of aggregates of proteins, possibly including the adenine nucleotide translocator, linked by disulfide bridges (12). The PT[1] is favored by a large number of agents, ranging from phosphate to radical chain initiators, and inhibited by others (for a tabulation see Ref. 8), including Mg^{2+} (13-16) and protons (17). The PT has been ascribed to the operation of a pore (17-19) with a diameter greater than 2.8 nm (11, 20), which is inhibited by cyclosporin A (21-24), a cyclic endecapeptide used as an immunosuppressant. As mentioned above, it has been proposed that this pore might not be present in the membrane under normal circumstances, arising instead by still obscure processes under the appropriate conditions (Ca^{2+} overload, radical processes). Indeed, a certain time lag is often needed, after the addition of the appropriate agents, for the membrane to become permeable. These processes leading to the "induction" of the pore may themselves be under the influence of various agents and must not be confused with the factors directly regulating pore activity. This paper is concerned only with this latter aspect.

Patch-clamp experiments on the mitoplast membrane (25-27) have shown that it harbors a giant, multicomponent, unselective (40) channel, with a conductance as high as 1.3 nanosiemens in 150 mM KCl, called here "mitochondrial megachannel" (MMC). We have recently shown that this channel is inhibited by cyclosporin A in the same concentration range in which the permeability transition is inhibited (27). This coincidence, and the large size of both the PT pore and the MMC, suggested that they might be the same species. Here we report that the MMC is activated by calcium and inhibited in a competitive manner by other divalent cations. Furthermore, cyclosporin A also competes with Ca^{2+} (and vice versa), and the activity of the MMC is sharply pH-dependent in the physiological range. These observations find a close correspondence in the effects of these agents on the induction of the permeability transition of mitochondria, studied by following mitochondrial volume variations, as detailed in the accompanying paper (28). A portion of this work has already been presented in abstract form (29).

MATERIALS AND METHODS

Rat liver mitochondria were isolated by standard centrifugation protocols, using 0.25 M sucrose, 0.1 mM EGTA, 10 mM Tris/Cl, pH 7.4, as the isolation medium throughout the procedure. Experiments were carried out as previously described (26, 27). Mitoplasts were prepared by osmotic shock with 30 mM Tris/Cl, followed by washing with the medium (see below). They were therefore exposed to 0.05 or 0.1 mM $CaCl_2$ for a few (normally 3-15) minutes before the high resistance seal was established. MMC induction presumably took place during these preliminary phases of the experiments. The voltage protocol consisted in the application of 1- or 2-s voltage pulses of alternating sign, separated by short intervals at zero potential. The filter corner frequency was 5 kHz, and the data were recorded in digital form (sampling frequency, 22 kHz). Experiments were conducted in symmetrical 150 mM KCl, 0.1 or 0.05 mM $CaCl_2$, 20 mM Hepes/K^+, pH 7.2. Both the mitoplast-attached and the excised patch configuration were used. Results were qualitatively the same in the two cases. Additions were made by injecting microliter amounts of

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[1] The abbreviations used are: PT, permeability transition; PTP, permeability transition pore; MMC, mitochondrial megachannel; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

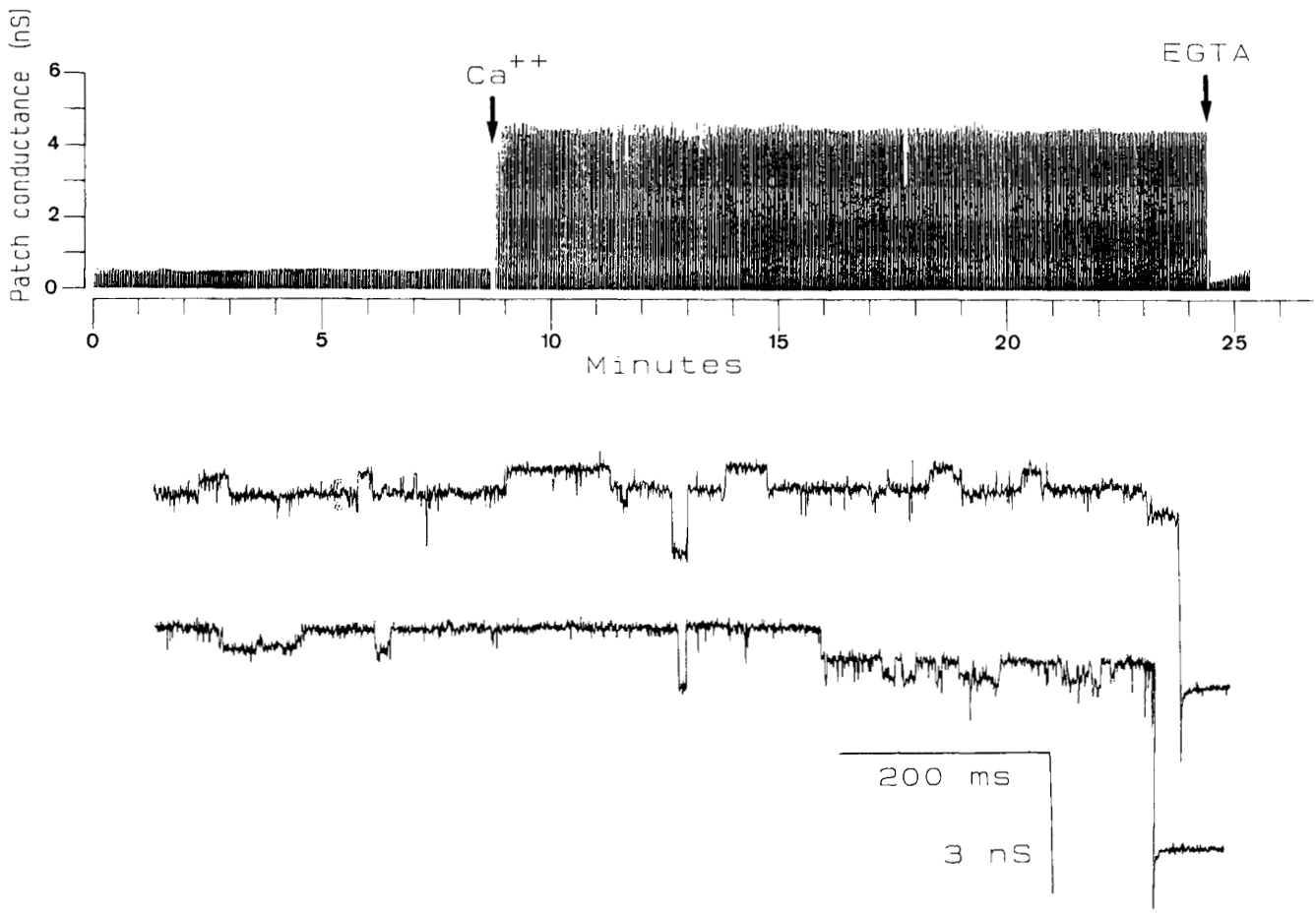

FIG. 1. **Ca^{2+} elicits MMC activity.** *Top,* the total (leaks not subtracted) conductance of an excised patch, averaged over 0.82-s intervals, *versus* time. *Arrows,* additions of 1.1 mM $CaCl_2$ and 2.8 mM EGTA, respectively. The chamber contained approximately 0.9 ml of medium. *Bottom,* current records exemplifying channel activity after Ca^{2+} addition. Voltage, 30 mV; digital sampling frequency, 5 kHz. The transitions at the extreme *right* are due to the voltage and current step to zero at the end of the voltage pulse (notice capacitive current spikes).

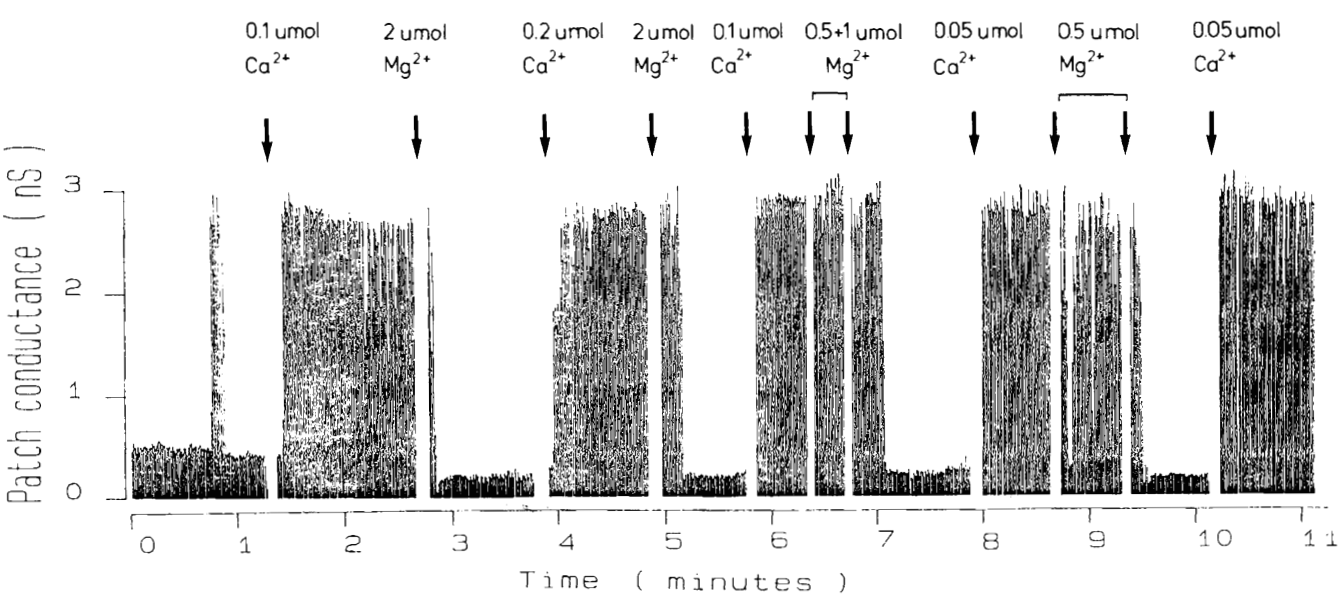

FIG. 2. **Mg^{2+} inhibits MMC activity in a competitive manner.** Plot of the total patch conductance, averaged over 0.82-s intervals, *versus* time. Mitoplast-attached configuration. $CaCl_2$ and $MgCl_2$ (amounts indicated, each *arrow* represents one addition) were added as 10 or 100 mM solutions, respectively. The patch chamber initially contained 0.88 ml of standard medium (with 0.1 mM $CaCl_2$). The addition of $CaCl_2/MgCl_2$ counteracts the inhibitory/activating effect of the previous addition. Voltage, ±20 mV.

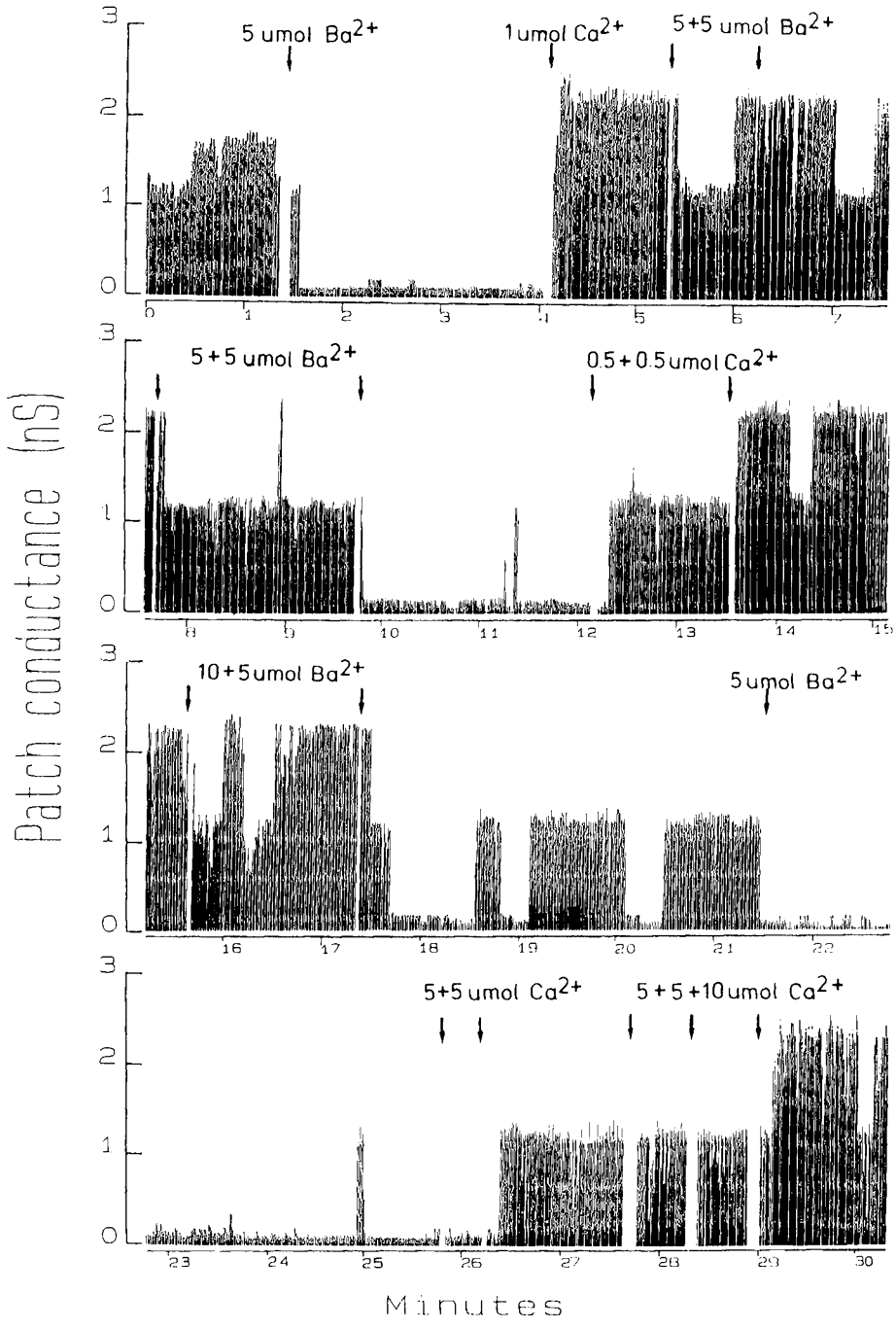

FIG. 3. **Competitive inhibition by Ba^{2+}.** Plot of the total conductance (averaged over 0.82-s intervals) of a mitoplast-attached patch *versus* time. $CaCl_2$ and $BaCl_2$ (amounts indicated) were added as 100 mM solutions. The patch chamber initially contained 0.77 ml of standard medium. Voltage, ±20 mV.

solutions into the patch chamber (containing 0.8–1 ml) with a microsyringe. After each addition the bath contents were completely mixed over 5–10 s. pH changes involved the exchange of the medium in the patch-clamp chamber by three sequential withdrawals and additions of 8–900-μl volumes. The pH and $[Ca^{2+}]$ of medium aliquots taken from the patch chamber during these operations were measured *a posteriori* ($[Ca^{2+}]$ by atomic adsorption spectrometry). Cyclosporin A was kindly provided by Dr. Roemer of Sandoz Ltd.

RESULTS

Activation by Ca^{2+}—About one-third of all the mitoplast membrane patches we have examined so far by the patch-clamp technique (in symmetrical standard medium) exhibited no activity upon application of voltage pulses. Yet, in 65% of these cases the addition of more $CaCl_2$ (submillimolar or millimolar range) to the patch chamber resulted in the nearly instantaneous appearance of the typical current patterns due to the activity of one or more MMCs. Addition of excess EGTA or EDTA caused the activity to stop abruptly. Fig. 1 presents one of these experiments; an increase in bath $[Ca^{2+}]$, and therefore in the $[Ca^{2+}]$ at the membrane matrix side (see "Discussion"), induced an immediate and lasting increase in the patch conductance, due to the opening of three megachannels. The current traces show that after the addition of Ca^{2+} the increased current was conducted by *bona fide* channels and was not due to increases in leak conductance. That the channels elicited by the Ca^{2+} addition were MMCs, and not other species, was indicated by the size of the conductance steps, by the inhibitory effect of cyclosporin A (see below, Fig. 4), and by the typical (26) voltage dependence of the conductances; 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). Ca^{2+} concentration was not the only factor controlling MMC activity in the experimental system; the

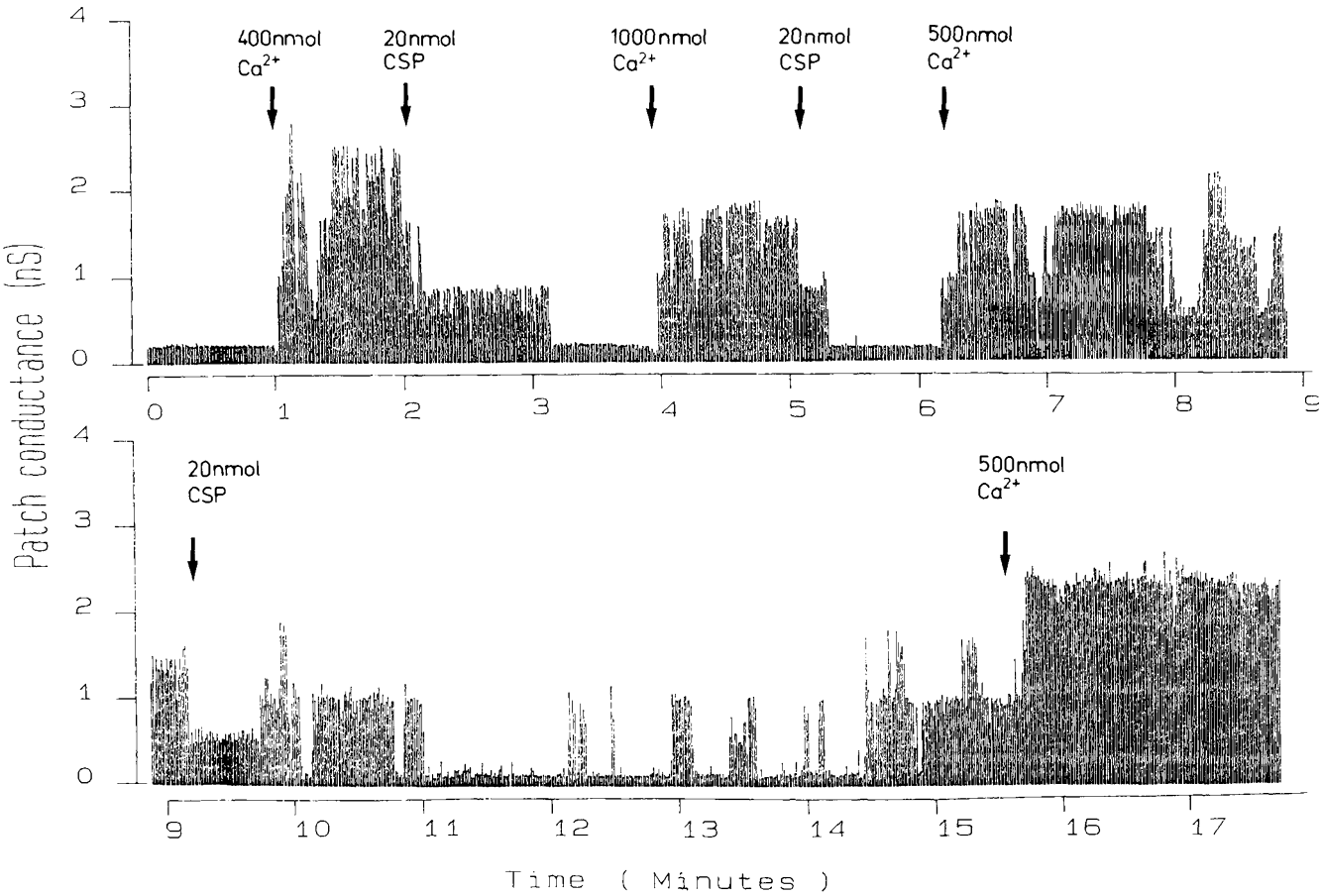

FIG. 4. **Competition between Ca^{2+} and cyclosporin A.** The total conductance (averaged over 0.82-s intervals) of a mitoplast-attached patch plotted *versus* time. $CaCl_2$ and cyclosporin A (*CSP*, amounts indicated) were added as 100 and 2 mM solutions. Initial volume of standard medium in the patch chamber, 0.85 ml.

open probability of MMCs in membranes bathed by the standard medium (see "Materials and Methods") varied from patch to patch. Furthermore, the proportion of inactive patches sometimes differed from one preparation of mitochondria to another. Recently Kinnally *et al.* (30) have reported that the likelihood of observing MMC activity in a patch is increased by the presence of Ca^{2+} (micromolar concentrations) during isolation of the mitochondria. In our case the mitochondria were prepared in the presence of 0.1 mM EGTA.

Inhibition by Divalent Cations—Fig. 2 shows that Mg^{2+} inhibited MMC operation (as observed in 15 out of 18 experiments). After inhibition by an addition of Mg^{2+}, MMC activity in the patch could be rekindled by increasing the concentration of Ca^{2+} in the medium. In turn, a further increase in the concentration of Mg^{2+} caused renewed inhibition, and so forth in cycles, apparently without limits, as exemplified in Fig. 2. In our experiments, the MMC open probability was nearly abolished at $[Mg^{2+}]/[Ca^{2+}]$ ratios of roughly 15, while it was close to the limiting value of 1 at ratios of approximately 5. The facts that (*a*) the channel activity depended on the relative (as opposed to absolute) concentrations of the two ions and (*b*) the activation/inhibition cycles could be carried out over a large (up to several millimolar) concentration range suggest that the modulation involves competition between the ionic species for one (or more) binding site(s).

The behavior of the other divalent cations tested, *i.e.* Mn^{2+}, Ba^{2+}, and Sr^{2+}, resembled that of Mg^{2+}. The competition is illustrated in Fig. 3 in the case of Ba^{2+}. Nearly complete inhibition of the MMC was observed at $[M^{2+}]/[Ca^{2+}]$ ratios of roughly 10, 20, and 30 for Mn^{2+}, Ba^{2+}, and Sr^{2+}, respectively, while the channel was mostly open at ratios of about 1, 8, and 4, respectively. These values are intended to provide only the merest indication of the ranges involved.

Competition between Ca^{2+} and Cyclosporin A—We have recently shown that cyclosporin A inhibits MMC activity in the submicromolar concentration range (27). It was of interest to check whether inhibition was influenced by the concentration of Ca^{2+} in the system or whether it involved a completely independent effector site. In experiments such as the one shown in Fig. 4, we followed the same experimental approach used with divalent cations (Figs. 2 and 3). As illustrated in the figure, a competitive pattern characterized the inhibition by cyclosporin A as well; additions of Ca^{2+} or cyclosporin A counteracted the effects of the preceding addition of the competitor, without identifiable limits. It must be noted that neither the divalent cations tested nor cyclosporin A inhibited a 107 picosiemens conductance with a tendency to close at negative potentials, presumably the "IMM" channel of Sorgato *et al.* (31), which accounts for part of the base-line conductance in Figs. 1–5.

Modulation by pH—The modulation of the MMC by protons is illustrated by Fig. 5. The channel turned out to be under steep pH control in the physiological range. With 100 μM medium Ca^{2+}, shifts of the bath pH from 7.20 to 6.8 caused the total inhibition of MMC activity in 10 out of 12 experiments. Nearly complete inhibition could be achieved in all cases by lowering the pH to 6.5 (Fig. 5). Remarkably, the inhibition caused by a decrease in pH could be overcome by an increase in $[Ca^{2+}]$, and it could be reinstated by a shift to

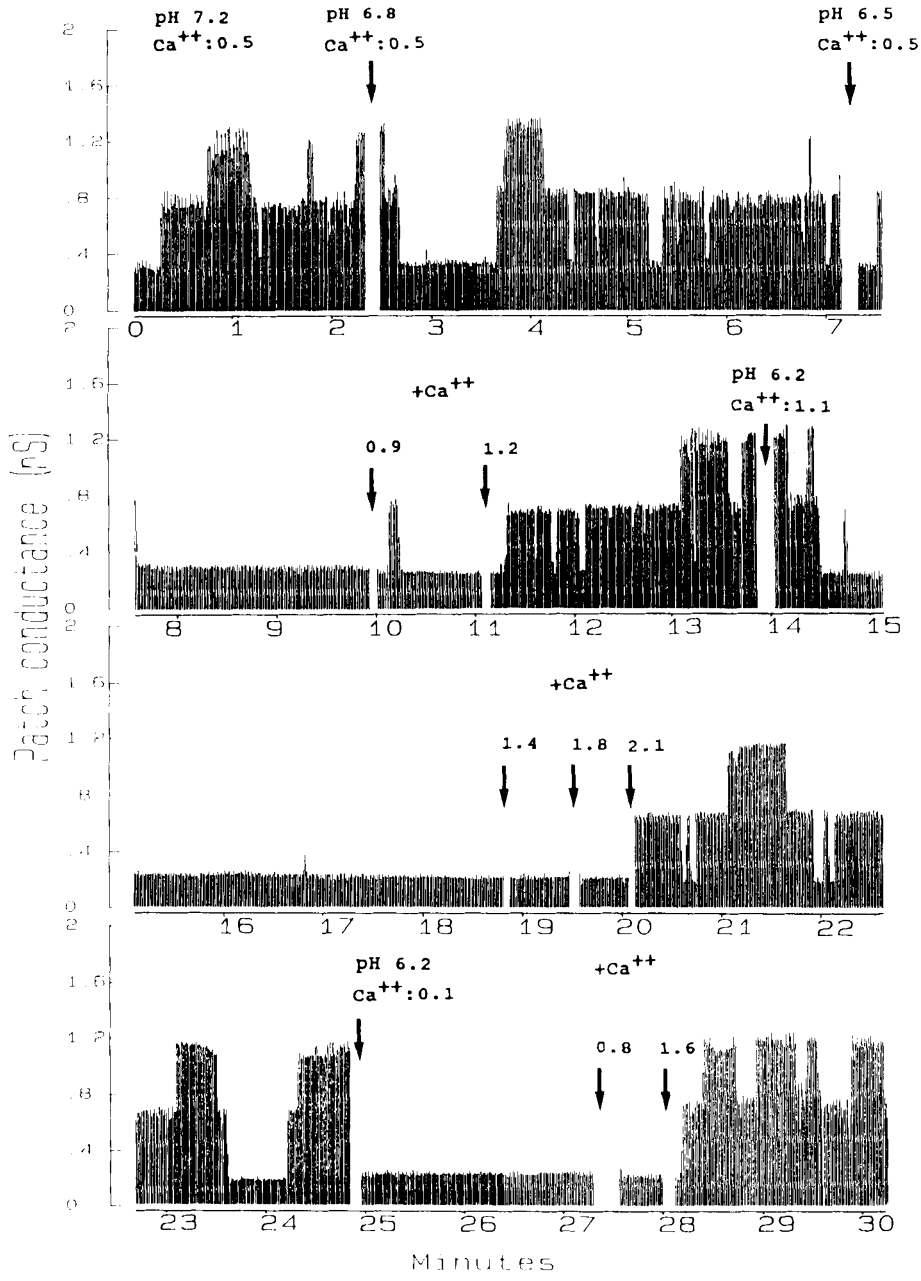

FIG. 5. **Ca^{2+}-reversible inhibition by acidification.** The total patch conductance (mitoplast-attached) was plotted as in the other figures. *Arrows* indicated either medium changes in the patch chamber (the new pH and millimolar Ca^{2+} concentration are specified) or $CaCl_2$ additions to give the Ca^{2+} concentration (millimolar units) specified. In this experiment a shift from pH 7.2 to 6.8 did not cause complete inhibition of the activity, which was, however, quenched almost completely at pH 6.5 ($[Ca^{2+}]$, 0.5 mM throughout). Increasing $[Ca^{2+}]$ to 1.2 brought back a high level of activity, which was blocked by a shift to pH 6.2. A further increase in the Ca^{2+} concentration, to 2.1 mM, caused the activity to reappear. When $[Ca^{2+}]$ was reduced to 0.1 mM the channels were, as expected, inhibited. Finally, they could be induced to function again by again increasing the Ca^{2+} level. Voltage, ±20 mV. In this experiment the "half-conductance" level (see text) was uncharacteristically prominent. Notice also the improvement in base-line conductance during the experiment.

even lower pH values (at the same $[Ca^{2+}]$).

Substates and Kinetic Behavior—Our recordings (26, 29) (Fig. 6) indicated that the MMC possesses a major substate with a conductance close to one-half of the highest level. This substate was generally visited only briefly; it was most often reached via partial closures from the fully open state, or it appeared for a few milliseconds as an intermediate level during some transitions from the fully open to the fully closed state. While the channel resided in this substate, the current often flickered rapidly, indicating that the channel switched from the (partial) open state to the closed state and back. Flickering was much less pronounced in the fully open state. In the experiment of Fig. 6, the mean channel open time was 100 times longer when the channel was in the full conductance state than when it resided in the "half-conductance" state.

Activation and inhibition phenomena, brought about by increases in the concentration of Ca^{2+} or of competing cations, respectively, were not, under the experimental circumstances employed in this work, gradual phenomena involving progressive increases in the mean open time and decreases in the mean closed time or vice versa. Rather, they tended to have an all-or-nothing character, with relatively small (percentage-wise) increases in the concentrations of the effectors causing drastic and lasting changes of the open probability. This can be appreciated in Figs. 1–5, in which recognizable (*i.e.* rather stable) patch conductance levels are induced by Ca^{2+} or the inhibitors. These levels result from the presence of a few channels which remain in a given conductance state (closed, "full," or "one-half") for seconds or minutes at a time. This behavior was responsible for the limited success of our attempts to determine titration curves for channel activity as a function of effector concentration.

DISCUSSION

The positive modulation of MMC activity by Ca^{2+} and its inhibition by other divalent cations, protons, and by cyclosporin A, described above, find a match in the observations on the permeability transition (7–9, 28), strongly suggesting

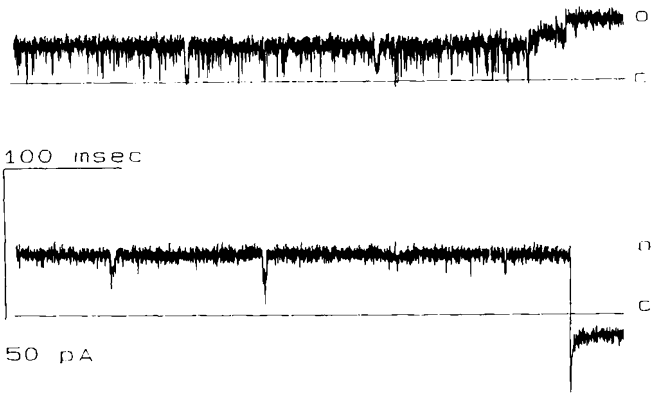

FIG. 6. **The kinetic behavior of the "one-half" and "fully open" conductance states.** An exemplificative trace segment from the same experiment as Fig. 5, showing both types of behavior by the same channel. Continuous trace. Bandwidth, 5 kHz; digitizing frequency, 20 kHz; voltage, +20 mV (transition to zero potential at the end). *c*, closed; *o*, open.

that the PT is indeed due to the operation of a channel, which can be identified with the MMC observed in patch-clamp experiments. Support for this identification has also recently been derived from the lack of selectivity by the channel, the calculated minimum pore size of its maximal conductance state, and by the inhibitory effect of ADP (40). We propose therefore that the identity of the MMC and PTP be taken as a credible working hypothesis. The agents listed above affect both the operation of the MMC, observed on a millisecond time scale, and the lag time of PTP "induction," on a time scale of minutes (28). This may indicate that a common mechanistic step is involved in pore induction and in the open/closed transitions of the channel. It should also be noted that in experiments performed on mitochondrial populations, the PTP, once induced, responds rapidly to these effectors (17). The properties and behavior of the channel are quite reproducible in our experiments. This means that the MMC either is constitutively present in the membrane or it is formed from well defined precursors by reproducible processes.

The effects of all the modulators tested must be assigned to events occurring on the matrix side of the inner mitochondrial membrane. The added ions or molecules would be expected to reach the cytoplasmic side of the patch, sealed off by the pipette rim, only with difficulty. Furthermore, once on the cytoplasmic side they would diffuse away into the pipette interior. If the modulating site(s) were situated on the cytoplasmic (pipette) face of the membrane, one would expect additions of inhibitors to the bath to produce either no effect or a rapid alternation of the channel between closed (bound inhibitor) and open states. This latter behavior would occur because binding of an inhibitor would close the channel, thus preventing the arrival of more inhibitor molecules. The bound inhibitor would then unbind and diffuse away into the pipette, the channel would open, some inhibitor molecules would therefore reach the inhibitory site, the channel would close again, and so forth. This behavior was not observed (see below). High concentrations of the added ions could develop instead on the matrix side of excised patches as well as of pipette-attached mitoplasts. In the latter case the ions could gain access to the matrix side via the operating MMCs and/or via the Ca^{2+} uniporter. Work on the permeability transition has also suggested a matrix side location for the Mg^{2+} effect (13, 14) as well as for the Ca^{2+}-binding regulatory site (8).

The competitive behavior of divalent cations, protons, and cyclosporin A is in agreement with the observations on the permeability transition (17, 28). The fact that Sr^{2+} and Mn^{2+} act as competitive inhibitors of channel operation explains why they cannot substitute for Ca^{2+} itself as activators or inducers of the pore. It also might help to explain observations such as Mn^{2+} protection of Ca^{2+}-induced ATP hydrolysis (32) or inhibition by Sr^{2+} of Ca^{2+}-induced Ca^{2+} release (33). In the case of divalent cations, it is logical to think in terms of fully competitive inhibition, with Ca^{2+} and the other cations vying for the same binding site, where they would exert opposing effects on the open probability. In the case of protons it might be more plausible to envision a partially competitive interaction; protonation at a distinct site would reduce the affinity of the divalent cation site. This view is in agreement with the observations of Haworth and Hunter (17), who reported a steep increase in the K_m for Ca^{2+} with increasing $[H^+]$.

The same authors (17) also reported cooperative binding of Ca^{2+} to the "trigger site," with a Hill coefficient close to 2, suggesting the presence of two interacting binding sites. There also appear to be two sites for ADP (34). Our observations (26) (Fig. 6 and "Results" section) on the presence and behavior of an approximately half-maximal conductance state may be integrated with these results to yield a tentative model of a channel composed of two units, each with binding sites for divalent cations, protons, and ADP, and capable of conducting current up to the ~500 picosiemens level. Activation and inactivation of the two units would be cooperative phenomena, with the transition of one facilitating a similar transition for the second, to give a stabilized state. This feature would explain the tendency of the full conductance channel to be modulated over narrow effector concentration ranges. This model must be regarded as a first approximation. Complexities are introduced by the presence of interacting binding sites for different species, by the observation of numerous other substates, and by the fact that the "half-channel" conductances are often lower or higher than one-half the maximal (1–1.3 nS) conductance. It is remarkable that a mitochondrial channel resembling a "one-half conductance" has been detected and studied in "dip-tip" experiments (35, 36). This channel has been suggested to be involved in protein translocation. A PT-dependent, cyclosporin-sensitive release of mitochondrial matrix proteins has been reported (37).

The location of the modulatory sites for the various effectors remains to be determined, but the obvious possibilities are the pore complex itself or mitochondrial cyclophilin, the soluble cyclosporin-binding protein which has been considered as a possible mediator of the effects of the latter on the permeability transition (38, 39). We tend to favor a localization of the binding site(s) on the pore complex itself because we observed the same activation and competitive inhibition pattern in excised membrane patches, which would be expected to have lost most of the soluble factors, and in patches belonging to whole, pipette-attached mitoplasts. Some recent results by McGuinness *et al.* (39), in particular the insensitivity of the prolyl isomerase activity of cyclophilin to Ca^{2+}, also argue against a direct involvement of cyclophilin in the modulation of MPC activity. Experiments are planned to check whether addition of cyclophilin to excised patches results in detectable changes in MMC activity.

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