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Evidence for a novel voltage-activated channel in the outer mitochondrial membrane

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Patch-clamp studies of the outer mitochondrial membrane indicate a voltage-dependent increase in conductance for potentials positive relative to the exterior of the mitochondrion. The time course of the conductance changes is consistent with an activation of channels. Voltage pulse experiments suggest that the activation phenomenon corresponds to assembly of the channels from subunits with disassembly occurring after recovery of the original conductance. Effects of temperature and concanavalin A on the voltage-induced conductances are also consistent with a channel assembly model.

Mitochondrial outer membrane; Ion channel; Patch clamp

1. INTRODUCTION

The mitochondrial outer membrane contains voltage-dependent anionic channels (called VDAC or mitochondrial porin) which can be isolated with detergents and reconstituted into phospholipid bilayers. The reconstituted channels exhibit multiple conductance states, switching from high to low conductance (4.5 to 2.5 nS in 1 M KCl) with applied voltages in the range of ± 20 to ± 50 mV [1,2]. As a different approach to the study of the permeability of the mitochondrial outer membrane, we are probing the electrical characteristics of the native membrane using patch-clamp techniques [3].

Mitochondrial outer membrane patches typically display resistances in the range 10–500 MΩ. While very low compared to those obtained with cell membranes (10–100 GΩ), these patch resistances are consistent with the expected high concentration of open channels in the mitochondrial outer membrane (e.g. 4000 VDAC polypeptides per μm^2 in the case of *Neurospora* [4]). Similar to the behavior of bilayers containing reconstituted VDAC, the conductance of the mitochondrial outer membrane patches decreases with increasing magnitude (−20 to −50 mV) of the negative voltage at which they are clamped [3]. However, in the range of potentials positive in relation to the pipette, patch conductances generally increase with potential. This behavior is a significant departure from that of VDAC-containing bilayers whose conductances decrease with increasing magnitude of potential regardless of polarity [1,2].

The present report demonstrates that the characteristics of the conductance increases at positive potentials are generally consistent with a model in which oligomeric channels are formed in the mitochondrial outer membrane.

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2. EXPERIMENTAL

The production of giant mitochondria (5–10 μm

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in diameter) in mice using cuprizone and their isolation have been described [5]. Typically the mitochondria were suspended in 0.30 osmolal sucrose, 10 mM KCl, 5 mM Hepes, pH 7.0, at 25°C.

Pipettes approx. 1.5 μm in tip opening were fused to the outer membrane of a giant mitochondrion [3]. The membrane patch on the pipette tip was then voltage-clamped and the resulting currents recorded [3]. The results described below were similar whether the patch excised or remained attached to the mitochondrion. The electronics used corrected for the in-series resistance corresponding to the microelectrode resistance at the appropriate temperature. For temperature studies a perfusion chamber of 1.1 ml capacity designed by Yamaguchi et al. [6] was used at a flow rate of 5 ml/min obtained with a Buchler variable-speed pump (Buchler Instruments, Fort Lee, NJ). Temperature was followed with a YSI model 42SL monitor (Yellow Springs Instruments, Yellow Springs, OH), with the probe submerged 3–5 mm from the tip of the pipette.

3. RESULTS AND DISCUSSION

3.1. *Kinetics of conductance changes*

In patches obtained with giant mitochondria, the increase in conductance that develops with time during a positive pulse of threshold or greater voltage is biphasic (figs 1,2A). A rapid rise in conductance is followed by a slower gradually accelerating increase of much greater magnitude. This conductance increase is reproducible and reversible and has been observed in both giant mitochondria from mice and fused vesicle preparations derived from the outer membrane of *Neurospora* mitochondria [3]. The threshold voltage varies from patch to patch and is typically in the range 1–50 mV. The second phase of the conductance change may take as long as several seconds to develop, depending on the patch and the applied voltage. In contrast, the return to a low-conductance state after the voltage pulse (monitored with a low background voltage) is much faster, taking only a fraction of a second (fig.2A). Experiments designed to determine the conductance change more precisely (not shown) indicate that they are within 50 ms.

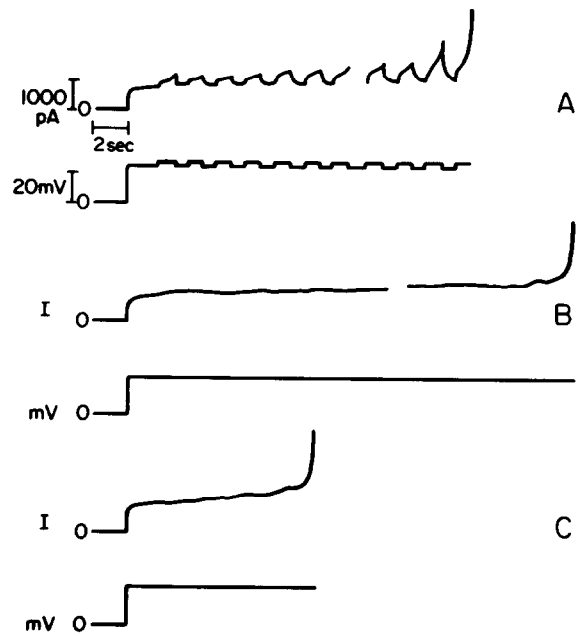

Fig.1. Effect of small voltage pulses superimposed on a larger pulse. (A) A 20 mV pulse and at regular intervals 2 mV pulses; (B) a 22 mV pulse alone; (C) a 25 mV pulse alone.

The long time course over which the conductance increases occur suggests an activation phenomenon, in particular one involving an assembly of multimeric channels from subunits dispersed in the membrane. Conductance changes attributed to channel formation by the α-toxin of *Staphylococcus aureus* in lipid bilayers exhibit a time dependence similar to that of our system and has led the authors to postulate an assembly model similar to ours [7]. The rapid decrease in conductance observed upon removal of the potential is expected for a channel assembly model, since detachment of a single subunit from a complex would lead to loss of channel function.

The state of the patch in relation to possible assembly phenomena can be probed with multiple voltage pulses applied during or after an initial pulse. For single pulses, a decrease in the duration of the latent period between the two conductance phases is seen when the pulse voltage is increased (cf. fig.1B and C). When a barely threshold voltage is applied, the system can be poised in the latent period for several seconds (fig.1B). Progressively larger conductance increases are induced

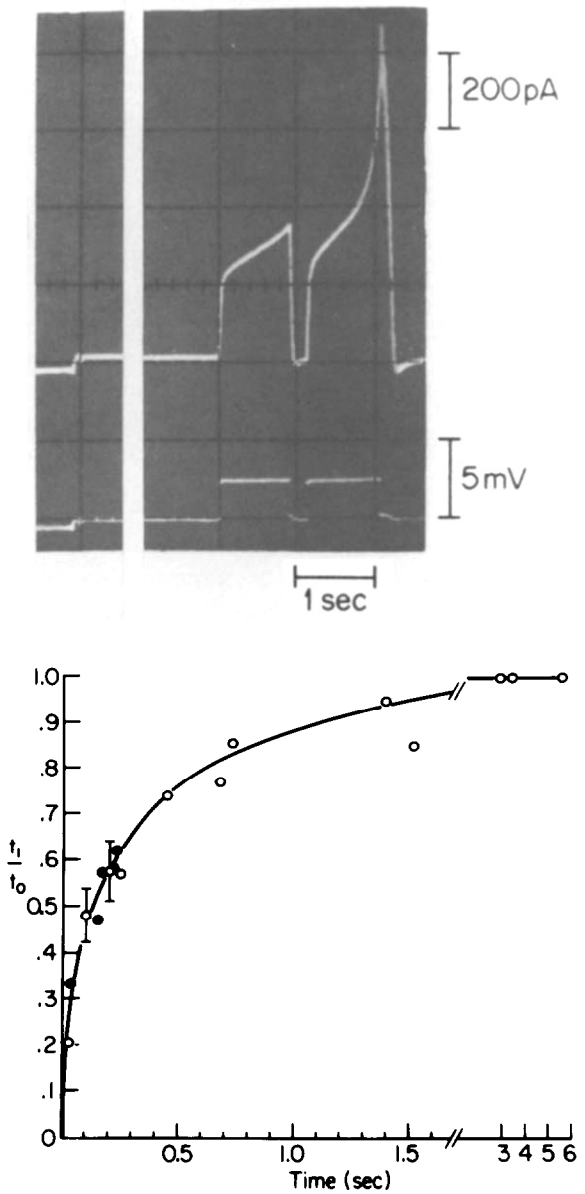

Fig.2. Effect of consecutive voltage pulses of the same magnitude on the conductance of outer mitochondrial membrane patches. (A) Oscilloscope recording of patch current response to two 2.5 mV pulses. A background voltage of 0.5 mV was introduced before the voltage pulses. (B) Plot of the time required to reach an end-point current of 1000 pA for the second pulse (t_1) over the equivalent value for the first pulse (t_0) as a function of the interval between the two pulses. These data are for a different patch from that in A. Both pulses were of 7.5 mV. Vertical lines represent SD ($N = 4$); (●) $N = 3$, (○) $N = 1$.

by probe pulses (2 mV in fig.1A) applied later in the latent period. This observation suggests a recruitment of subunits.

As shown in fig.2A, while the conductance recovery from an initial pulse is rapid, a second identical voltage pulse elicits a much greater conductance response. The results of similar double-pulse determinations using another patch are summarized in fig.2B. This is a plot of the time (t_1) required to reach a current of 1000 pA for a second voltage pulse as a function of the time interval between the identical pulses. The values of t_1 are normalized by dividing by t_0, the time to reach the same end-point current during the initial pulse. The total recovery time of the system corresponds to the time interval between pulses for which $t_0/t_1 = 1$, or about 2 s in fig.2B. These results show that the system possesses a 'memory', i.e. in terms of our model a second pulse reconstitutes the highly conducting channels from partially dissociated complexes. In this model, a memory requires a channel of at least three subunits and the recovery time corresponds to the time for disassembly of the channel.

3.2. *Temperature dependence of conductance changes*

The conductance changes elicited by a pulse of 4 mV across a patch perfused with a medium at the indicated temperatures are compared in fig.3. This figure shows that the magnitude of the conductance response increases with temperature between 5 and approx. 30°C. However, at approx. 30°C, the conductance decreases with temperature, and in this experiment the conductance increase does not occur at 35°C unless higher voltages (6 mV or more) are used. These effects are observed whether the experiment is carried out with increasing or decreasing temperature.

Many temperature studies have been carried out on the conductance of a variety of channels [7–16]. To our knowledge, a temperature dependence similar to that reported here has been observed in only two systems known to require assembly [15,16]. The conductance of phospholipid bilayers containing alamethicin multimeric channels [15] has a maximum at 23°C which is just below the phase transition temperature of the membrane phospholipid. This effect has been attributed to differences in the con-

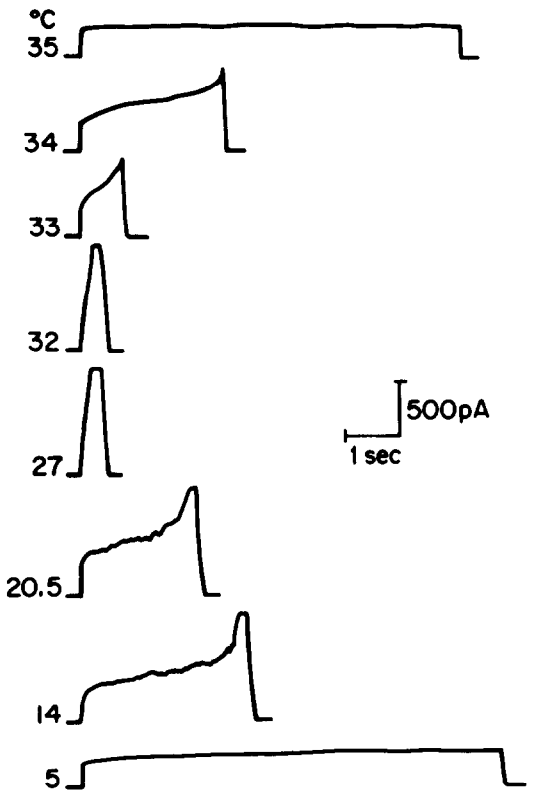

Fig.3. Effect of temperature on the conductance of a mitochondrial membrane patch. Pulses of 4 mV were delivered to the same patch while the preparation was perfused with incubation medium at the various temperatures (0°C) shown. The duration of the pulse in each case corresponds to the duration of the current records. The flattening of the curves indicates the maximum current which can be recorded by the system.

formation and/or lateral distribution of the alamethicin subunits in gel and liquid-crystalline bilayer phases. These differences may alter the ability of the monomers to aggregate into functional complexes. Similarly, an activity assay thought to reflect assembly of *S. aureus* α-toxin in rabbit erythrocytes shows a maximum at 30°C [16] which has been attributed to a change in membrane fluidity affecting α-toxin assembly. The temperature dependence of the conductance response observed in mitochondrial outer membrane patches (maxima about 30°C) supports the notion that an assembly of channels is involved in the observed conductance changes. Experiments are being planned to determine whether lipid phase transitions occur in the mitochondrial outer membranes of cuprizone-treated mice around the temperature at which the response of this membrane's conductance to positive voltages is similarly altered.

3.3. *Nature of membrane components involved in conductance changes*

Concanavalin A can aggregate proteins (in particular glycoproteins) which are free to diffuse in the plane of the membrane, as indicated in some cells by capping [17–19]. The addition of concanavalin A increases the effects of small positive voltages on the conductance of mitochondrial outer membrane patches (fig.4). This finding suggests that a partial pre-assembly of channel complexes occurs in the presence of concanavalin A. A glycoprotein (93 kDa) of unknown function is present in the outer membrane of rat liver mitochondria [20] and it might be expected to bind concanavalin A. The abundant pore-forming VDAC protein (30–35 kDa) also has an affinity for concanavalin A [21], although it is probably not a glycoprotein.

3.4. *Conclusions and conjectures*

Our results are generally consistent with a model in which the increases in conductance induced by

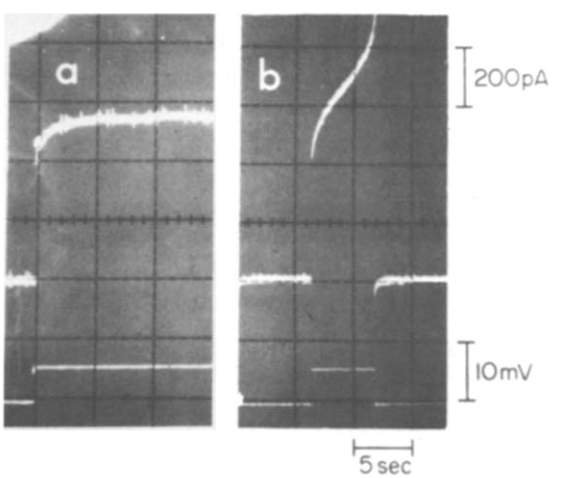

Fig.4. Effect of concanavalin A on the conductance of a mitochondrial membrane patch at 25°C. (a) The patch was subjected to a voltage pulse of 6 mV during perfusion with the usual medium. (b) The effect of the same voltage pulse after incubation for 15 min in the same medium containing 50 μg/ml of concanavalin A.

positive potentials correspond to the assembly of channels from subunits. A stronger case would be made if we had been able to demonstrate the stepwise opening of single channels. This is technically difficult for patches containing a large number of channels. However, we are not aware of any other single model which could explain the experimental observations described here.

Serious discussion of the possible biological significance of the novel mitochondrial outer membrane channels inferred from patch-clamp experiments requires further information. Singer et al. [22] have recently proposed a role for water-filled oligomeric channels in the transfer of polypeptide chains across membranes. Blumenthal et al. [23] have demonstrated a non-linear, voltage-dependent conductance increase in phospholipid bilayers containing receptors for asialoglycoprotein.

The biological signal for the opening of the putative channels also remains unclarified. A variety of effects could produce localized Donnan potentials sufficient to produce the conductance changes reported in this paper since in some mitochondrial outer membrane patches, as little as 1 mV produces a significant effect.

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