Activity of the Mitochondrial Multiple Conductance Channel Is Independent of the Adenine Nucleotide Translocator*  

(Received for publication, September 1, 1995, and in revised form, November 8, 1995)

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The functional relationship between the adenine nucleotide translocator (ANT) and the mitochondrial multiple conductance channel (MCC) was investigated using patch-clamp techniques. MCC activity with the same conductance, ion selectivity, voltage dependence, and peptide sensitivity could be reconstituted from inner membrane fractions derived from mitochondria of ANT-deficient and wild-type Saccharomyces cerevisiae. In addition, the MCC activity of mouse kidney mitoplasts was unaffected by carboxyatractysolide, a known inhibitor of ANT and inducer of a permeability transition. These results suggest that MCC activity is independent of ANT.

Patch-clamp experiments on mitoplasts (mitochondria with the inner membrane exposed) have detected a multiple conductance channel MCC (also referred to as mitochondrial megachannel, MMC), which is activated by calcium (1–6). This multistate channel activity is voltage-dependent and has a peak conductance of 1–1.5 nanosiemens (see Refs. 2 and 4 for recent reviews). Szabó and Zoratti (5) have proposed that the adenine nucleotide translocator (ANT) may be responsible for a permeability transition in mitochondria. Halestrap and Davidson (7) have postulated that the adenine nucleotide translocator (ANT) is intrinsic to a permeability transition. Recently, Zoratti and Szabó (8) have speculated on the possibility that MCC may be associated with a dimer of ANT plus other proteins.

The protein(s) responsible for MCC activity have not been identified. However, it is possible that MCC may be associated with a membrane transporter like ANT since many transporters display channel activity when reconstituted in bilayers and proteoliposomes. These transporters include the (Na+/K+)-ATPase, the chloroplast triose phosphate/phosphate translocatot, and members of the ATP binding cassette superfamily of transporters, e.g. cystic fibrosis transmembrane conductance regulator and multidrug-resistant transporters (8–14). In fact, channel activity similar to that of MCC has been reported in black lipid membranes fused with proteoliposomes containing protein fractions of ANT (15). Possible relationships between MCC and transporters can now be examined by using deletion mutants of mitochondrial transporters in yeast. Unfortunately, the other dominant inner mitochondrial membrane channel (mitochondrial centum picosomien channel) has not yet been detected in yeast (16).

In the present work, we have used patch-clamp techniques to compare the MCC activity in proteoliposomes reconstituted with mitochondrial inner membranes from ANT-deficient and wild-type strains of Saccharomyces cerevisiae. In addition, we have applied similar techniques to mouse kidney mitoplasts to see whether MCC activity is affected by carboxyatractysolide, which is a specific inhibitor of ANT (17).

Materials and Methods

Mitochondria, Mitoplast, and Proteoliposome Preparations—Mouse kidney mitochondria were isolated as described previously (18), and mitoplasts were prepared using the French press method (at 2000 p.s.i.) of Decker and Greenawalt (19). Mitochondria from S. cerevisiae strain M3 (wild type, graciously provided by M. Forte, Vollum Institute) and strain J L–1–3 (a triple mutant in which all three genes (AAC1, AAC2, AAC3) encoding mitochondrial ANT have been disrupted) (20) were isolated by a modification of the method of Daum et al. (21). In general, yeast channel activities were studied after reconstitution into proteoliposomes. Isolated yeast mitoplasts (prepared by French press) were osmotically lysed and spun on a sucrose gradient according to the method of Mannella (22). Pellets from the gradient spin were used as inner membranes, which were reconstituted into giant proteoliposomes using the dehydration-rehydration method of Criado and Keller (23) in 0.15 M KCl, 5 mM HEPES, pH 7.4, as described previously (6, 16). Inner membrane yields were ~10 and 2.5 mg of protein from overnight cultures of 20 g (2 liters) and 17 g (1.5 liters) of strains M3 and J L–1–3 cell pellets, respectively. Approximately 10% of each inner membrane preparation (0.25–1 mg of protein) was added to liposomes (~5 mg of Sigma type IV-S soybean L-α-phosphatidylcholine) for a typical reconstitution.

Patch Clamping—The procedures and conditions used for patch clamping mitoplasts and proteoliposomes are essentially the same as previously reported (18, 24). The reported voltage is that of the mitochondrial matrix in inside-out excised patches from mitoplasts, where V = V bath − V pipette. Voltages are reported relative to the bath for proteoliposome patches since the orientation of MCC (as indicated by voltage dependence and kinetics) is the same as in mitoplasts. Open probability is calculated as the fraction of the total time spent in the fully open state from amplitude histograms using the PAT program (Strathclyde Electrophysiological Software, courtesy of J. Dempster, University of Strathclyde, UK). All yeast experiments were done in 0.15 M KCl, 5 mM HEPES, 1 mM EGTA, 1.05 mM CaCl2, (10−5 M free Ca2+), pH 7.4. Selectivity was estimated from the reversal potential with a 5:1 KCl gradient as described previously (16). The level of MCC activity in mammalian mitoplasts was varied by exposure to calcium as in Ref. 3 and the patching media as indicated in Table I.

Effectors—Peptides were prepared by the Wadsworth Center’s peptide synthesis core facility on an Applied Biosystems 431A automated peptide synthesizer as described previously (6). The targeting peptides used were the amino termini of cytochrome oxidase subunit VI (YMLRAIFNVPVINTLRLR29) and subunit IV (YMLRSQRSIF-FKY113) of S. cerevisiae and subunit IV (YRAPRRLSIATTYYRVC-NAET22) of Neurospora crassa. The control peptide was the amino terminus from N. crassa voltage-dependent anion-selective channel (YAVAPFSDIASKSANDLNNK20). Carboxyatractysolide (Sigma) was included in the medium in the patch pipette (and usually the bath) at

*This study was supported by National Science Foundation Grant MCB9117658. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: MCC, multiple conductance channel; ANT, adenine nucleotide translocator.
concentrations of 5 or 10 \( \mu \text{M} \). The presence or absence of carboxyatractyloside was alternated every 3–5 patches within each preparation of mammalian mitoplasts.

Verification of Phenotypes by Western Blot Analysis—Western blot analysis was performed on both wild-type and ANT-deficient purified mitochondrial inner membranes. Samples (10 \( \mu \text{g} \) of protein) were boiled in SDS-polyacrylamide gel electrophoresis sample buffer, electrophoresed in 14% polyacrylamide gel, and blotted onto a polyvinylidene difluoride membrane (Novex, San Diego, CA). Samples were then probed with anti-ANT antibodies recognizing all three isoforms of ANT (graciously provided by J. Kolarov and I. Hapala) and visualized with a horseradish peroxidase-coupled second antibody (Pierce) with diaminobenzidine as a substrate (25).

RESULTS

MCC Is Present in ANT-deficient Yeast—Patch-clamp techniques were used to characterize the MCC activity in proteoliposomes containing mitochondrial inner membranes from a wild-type strain of yeast and a strain in which the three ANT genes were disrupted. Western blot analysis confirmed the absence of ANT in the inner membranes prepared from the later strain as shown in Fig. 1. Fig. 2 illustrates typical channel activity detected in proteoliposomes derived from the ANT-deficient strain (\( n = 8 \) patches). The peak conductance state (\(-1.1 \text{ nS} \)) and half-open substate are typical of MCC transitions observed in preparations from wild-type yeast (6, 16). MCC activity from the ANT-deficient strain is voltage-dependent, occupying near-peak conductance levels in the potential range between \(-40 \text{ mV} \) and \(+20 \text{ mV} \). At larger amplitude transmembrane potentials of either polarity, the channel tends to occupy lower conductance levels including a low noise closed state (occurring at \(-40 \text{ mV} \) in Fig. 2). This voltage-dependent behavior is the same as that of reconstituted MCC from wild-type strains of yeast (2, 6, 16).

No significant differences between the MCC activity of wild-type and ANT-deficient yeast were resolved. MCC activity of the ANT-deficient strain is slightly cation-selective (\( P_{\text{K}^+}/P_{\text{Cl}^-} \approx 6, n = 2 \), data not shown) like that of the wild-type strain (\( P_{\text{K}^+}/P_{\text{Cl}^-} \approx 6, \text{Ref. 16} \)). Peptides whose sequences target precursor proteins to the mitochondrial inner membrane specifically induce a transient blockade of MCC from the ANT-deficient strain (\( n = 7 \) patches) as previously reported for MCC from the wild-type strain (6) (Fig. 3). Furthermore, the frequency of recording MCC is about the same from proteoliposomes from the wild-type (75\%, \( n = 12 \) patches) and ANT-deficient (88\%, \( n = 8 \) patches) yeast strains. We also noted that the 50 picoSiemens channel activity often detected in wild-type yeast proteoliposomes (16) was also present in the ANT-deficient yeast proteoliposomes (data not shown).

Mammalian MCC Is Not Affected by Carboxyatractyloside, an ANT Inhibitor—Carboxyatractyloside stabilizes the nucleotide binding site of ANT on the cytoplasmic side of the inner membrane, thereby blocking the exchange of matrix ATP and cytoplasmic ADP (\( K_i \approx 10^{-9} \text{ M} \)) (17). The effect of this ANT inhibitor on MCC activity was determined in mouse kidney mitoplasts. As shown in Table I, the frequency of recording MCC was not changed by the presence of carboxyatractyloside under a variety of conditions. That is, carboxyatractyloside does not appear to activate MCC under low calcium conditions.

![Fig. 1. ANT is absent in AAC1, AAC2, AAC3 deletion mutant.](http://www.jbc.org/)

![Fig. 2. MCC activity is present in ANT-deficient yeast.](http://www.jbc.org/)
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...that normally favor quiescent MCC (3). Likewise, this ANT inhibitor does not appear to inhibit MCC activated by calcium. As shown in Table I, the seal resistance was unchanged by the presence of carboxyatractyloside, indicating that there was no masking of MCC in a stabilized open state. (If MCC were immobilized in an open state, resistance would be below 0.5–1 gigaohm). The studies were restricted to the potential range of \( \pm 30 \text{ mV} \) to avoid voltage activation of MCC (26). (In general, the presence of MCC in patches scored inactive was verified by activation of MCC by higher voltages (\( \pm 60–100 \text{ mV} \)) in the presence and absence of carboxyatractyloside.)

**DISCUSSION**

**ANT Is Not Responsible for MCC Activity**—The protein(s) responsible for MCC have not yet been identified but Zoratti and Szabó (4) have speculated that this channel activity might be associated with a dimer of the transport protein ANT (plus other proteins). Tikhonova et al. (15) detected channel activity similar to that of MCC in black lipid membranes fused with proteoliposomes containing purified fractions of ANT after mersalyl addition. In addition, mersalyl induced a high conductance pathway in the proteoliposomes that was not sensitive to carboxyatractyloside. Tikhonova et al. (15) suggest that mersalyl may modify ANT so that it can operate as a channel and suggest that ANT may function as a permeability transition pore-forming protein. Similarly, Dierks et al. (27) report mersalyl induces an ATP efflux from proteoliposomes co-reconstituted with ANT and the aspartate/glutamate carrier. This efflux was inhibited 50% by concentrations of carboxyatractyloside that also eliminated ANT-mediated nucleotide exchange (27) but had no effect on aspartate efflux.

We used a molecular genetics approach to test the hypothesis that ANT was responsible for MCC activity. This has advantages over biochemical approaches, which may be compromised or confounded by trace contamination by channel proteins that are detectable with patch-clamp techniques. Channel activity with the same conductance, ion selectivity, voltage dependence, and peptide sensitivity as MCC was reconstituted from inner membrane fractions derived from mitochondria of ANT-deficient and wild-type yeast strains. These results strongly indicate that ANT is not responsible for MCC. This conclusion was supported by experiments which showed carboxyatractyloside, a known inhibitor of ANT and an activator of a permeability transition, had no effect on mammalian MCC. However, this work does not eliminate the possibility that ANT may have channel activity unrelated to MCC.

**MCC and the Mitochondrial Permeability Transition**—A permeability transition can be induced in mitochondria by calcium and a trigger, e.g. phosphate. Similarly, carboxyatractyloside is known to induce a permeability transition in mitochondria (7, 28). Furthermore, a model has been proposed in which the carboxyatractyloside-induced permeability transition (5) since they share many characteristics including estimated pore size and pharmacology (see Refs. 2 and 4 for recent reviews). However, it is not likely MCC is associated with the permeability transition induced by carboxyatractyloside since, in this study, MCC activity was insensitive to this transition inducer. This apparent conflict may, however, be resolved since studies indicate that the induction of the permeability transition by carboxyatractyloside may be indirect by modifying adenine nucleotide levels (4, 29).

The diversity of permeability transition effectors leads one to speculate that more than one high permeability pathway may be involved in these transitions. For example, cyclosporine inhibits the calcium-induced transition but not the butylated hydroxytoluene-induced transition (30). If multiple pathways exist, our findings suggest MCC is not responsible for the permeability pathway induced by carboxyatractyloside.

Conclusions—Reconstituted MCC activity is the same in yeast lacking all three ANT genes as it is in the wild type. In addition, mammalian MCC is not affected by carboxyatractyloside, a known inhibitor of ANT. These findings indicate that...
the inner membrane translocator ANT is not responsible for the multiple conductance channel activity.

Acknowledgments—We thank Dr. James Dias and the Wadsworth Center’s peptide synthesis core facility for synthesis of peptides. We thank Dr. Michael Forte for the yeast strain M-3 and Drs. Jordan Kolarov and Ivan Hapala for the ANT antibodies. We thank Dr. Enrico Cabib for his support of T. D. at NIDDK-LBM, NIH. We thank Drs. Patricia Sokolove, Richard Zitomer, Henry Tedeschi, and especially Carmen Mannella for support, discussions, and review of this manuscript.

REFERENCES
1. Kinnally, K. W., Zorov, D. B., and Antonenko, Yu. (1992) J. Bioenerg. Biomembr. 24, 99–110
2. Kinnally, K. W., Lohret, T. A., Campo, M. L., and Mannella, C. A. (1996) J. Bioenerg. Biomembr. 28, in press
3. Kinnally, K. W., Zorov, D. B., Antonenko, Yu., and Perini, S. (1991) Biochem. Biophys. Res. Commun. 176, 1183–1188
4. Zoratti, M., and Szabó, I. (1995) Biochim. Biophys. Acta 1241, 139–176
5. Szabó, I., and Zoratti, M. (1992) J. Bioenerg. Biomembr. 24, 111–117
6. Lohret, T. A., and Kinnally, K. W. (1995) J. Biol. Chem. 270, 15950–15953
7. Halestrap, A. P., and Davidson, A. M. (1990) Biochem. J. 258, 153–160
8. Last, T. A., Gantzer, M. L., and Tyler, C. D. (1983) J. Biol. Chem. 258, 2399–2404
9. Mironova, G. D., Bocharnova, N. I., Mirsalikhova, N. M., and Mironova, G. P. (1986) Biochim. Biophys. Acta 861, 224–236
10. Schwarz, M., Gross, A., Steenkamp, T., Flugge, U. I., and Wagner, R. (1994) J. Biol. Chem. 269, 29481–29489
11. Thevenod, F., Anderle, I., and Schulz, I. (1994) J. Biol. Chem. 269, 24410–24417
12. Abraham, E. H., Prat, A. G., Gerweck, L., Seneveratne, T., Arced, R. J., Kramer, R., Guidotti, G., and Cantiello, H. F. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 312–316
13. Kunzelmann, K., Slotki, I. N., Klein, P., Koslowsky, T., Ausiello, D. A., Greger, R., and Cabantchik, Z. I. (1994) J. Cell. Physiol. 161, 393–406
14. Zhang, J. J., and Jacob, T. J. (1994) Am. J. Physiol. 267, C1095–C1102
15. Tikhonova, I. M., Andreyev, A. Yu., Antonenko, Yu. N., Kaulein, A. D., Komarov, A. Yu., and Skulachev, V. P. (1994) FEBS Lett. 337, 231–234
16. Lohret, T., and Kinnally, K. W. (1995) Biophys. J. 68, 2299–2309
17. Klingenberg, M. (1980) J. Membr. Biol. 56, 97–105
18. Campo, M. L., Kinnally, K. W., and Tedeschi, H. (1992) J. Biol. Chem. 267, 8123–8127
19. Decker, G. L., and Greenawalt, J. W. (1977) J. Ultrastruct. Res. 59, 44–56
20. Drgoň, T., Sabová, L., Nelson, N., and Kolarov, J. (1991) FEBS Lett. 289, 159–162
21. Daum, G., Böhni, P., and Schatz, G. (1982) J. Biol. Chem. 257, 13028–13033
22. Mannella, C. A. (1982) J. Cell Biol. 94, 680–687
23. Criado, M., and Keller, B. U. (1987) FEBS Lett. 224, 172–176
24. Kinnally, K. W., Zorov, D. B., Antonenko, Yu., Snyder, S., and Tedeschi, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1374–1378
25. Smith, J. A. (1995) in Current Protocols in Molecular Biology (Ausbel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Sieden, J. G., Smith, J. A., Struhl, K., and Janssen, K., eds) pp. 10.2–10.8, John Wiley & Sons, Inc., New York
26. Zorov, D. B., Kinnally, K. W., Perini, S., and Tedeschi, H. (1992) J. Biol. Chem. 267, 119–124
27. Diener, T., Salentin, A., Heberger, C., and Krämer, R. (1990) Biochim. Biophys. Acta 1028, 268–280
28. Gunter, T. E., and Pfeiffer, D. R. (1990) Am. J. Physiol. 27, C755–C786
29. Novgorodov, S. A., Gudz, T. I., Brierley, G., and Pfeiffer, D. R. (1994) Arch. Biochem. Biophys. 311, 219–228
30. Sokolove, P. M., and Haley, L. M. (1996) J. Bioenerg. Biomembr. 28, in press
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J. Biol. Chem. 1996, 271:4846-4849.
doi: 10.1074/jbc.271.9.4846

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