Journal of Bioenergetics and Biomembranes, Vol. 13, Nos. 5/6, 1981

Activation of Potassium-Dependent H^+ Efflux from Mitochondria by Cadmium and Phenylarsine Oxide[1]

D. Rao Sanadi,[2] James B. Hughes,[2] and Saroj Joshi[2]

Received March 24, 1981

Abstract

Additon of Cd^{2+} or phenylarsine oxide (PhAsO) to respiring rat liver mitochondria results first in acidification of the medium (H^+ efflux) followed by disappearance of H^+ (discharge of the pH gradient or uncoupling). The first phase of H^+ efflux is dependent upon the presence of K^+ in the medium, and is not seen in the presence of valinomycin, which is consistent with the conclusion that H^+ efflux is linked to membrane potential-dependent uptake of K^+. These effects are abolished by low levels of 2,3-dimercaptopropanol but potentiated by excess of 2-mercaptoethanol, showing involvement of a dithiol type of group in the response. Mersalyl produces only the H^+ efflux, and subsequent addition of Cd^{2+} or PhAsO produces collapse of the ΔpH.

Key Words: Cadmium; phenylarsine oxide; mitochondria; H^+ translocation; potassium ion; uncoupling; dithiol; monothiol.

Introduction

Earlier work had revealed evidence for a hydrophobic dithiol type of functional site in the oxidative phosphorylation process. The conclusions were based on the following findings: (a) Cadmium ion (Cd^{2+}), arsenite in the presence of BAL, and organic arsenicals uncoupled oxidative phosphorylation and released respiratory control (Jacobs et al., 1956; Fluharty and Sanadi, 1961, 1962, 1963). (b) They stimulated the mitochondrial latent ATPase, and the stimulated ATPase was inhibited by oligomycin. (c) These uncoupling effects were inhibited by low levels of a dithiol but not by much higher levels of monothiols. In fact, monothiols potentiated the uncoupling effects of

[1]Abbreviations: BAL, British Anti-Lewisite or 2,3-dimercaptopropanol; 2-ME, 2-mercaptoethanol; PhAsO, phenylarsine oxide; FCCP, carbonylcyanide trifluoromethoxyphenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

[2]Cell Physiology Department, Boston Biomedical Research Institute, 20 Stanford Street, Boston, Massachusetts 02114, and Department of Biological Chemistry, Harvard Medical School.

0145-479X/81/1200-0425$03.00/0

organic arsenicals. The reaction site in mitochondria thus competed effectively with excess monothiols for these compounds and showed roughly the same affinity as did dithiols.

More recently, we found that Cd^{2+} and PhAsO caused discharge of the mitochondrial transmembrane H^+ gradient generated by substrate oxidation (Sanadi *et al.*, unpublished). 2-Mercaptoethanol potentiated the effect but BAL at low concentrations prevented the discharge. During the course of these studies, a transient increase in the H^+ gradient following the addition of Cd^{2+} and PhAsO was observed. This communication reports properties of this initial phase of the response.

Materials and Methods

Rat liver mitochondria were prepared in a mannitol–sucrose medium as described by Johnson and Lardy (1967). They showed a respiratory control ratio of 3 to 4 with succinate as substrate. Protein determination was by the biuret method in the presence of 0.33% deoxycholate using bovine serum albumin as the standard (Jacobs *et al.*, 1956). Oxygen uptake was measured with a Clark electrode (Rank Brothers, Bottisham, Cambridge, England) and pH was monitored using a combination glass electrode (Ingold Electrodes, Inc., Andover, Massachusetts) and a Keithley electrometer (Model 610C). The changes were followed on a dual channel strip chart recorder (Servariter II, Texas Instruments).

The reaction medium was 0.25 M sucrose, 0.02 M KCl or NaCl as specified, and 3 mM HEPES for the experiments with PhAsO or 3 mM phosphate for experiments involving Cd^{2+}. The substrates were 2 mM succinate or 2 mM 3-hydroxybutyrate plus 50 μM NAD^+. The other reagents are specified under the figures or tables.

Valinomycin, oligomycin, and mersalyl were supplied by Sigma Chemical Co. FCCP was a gift from Dr. P. G. Heytler. PhAsO was obtained from Aldrich Chemical Co.

Results

When PhAsO is added to respiring rat liver mitochondria, there is a slow decrease in pH or extrusion of hydrogen ions from mitochondria. The process shows a lag, and is followed by a disappearance of H^+ from the medium or uptake of H^+ (Fig. 1, trace 1) corresponding to discharge of the transmembrane pH gradient. The response is dependent on the concentration of the arsenical and is optimal at 5 μM. The effect of PhAsO is similar in the

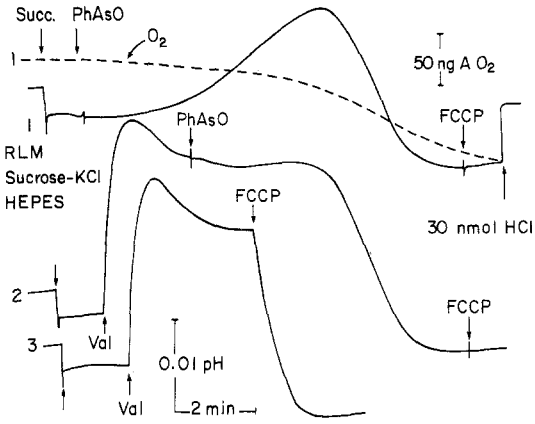

Fig. 1. H^+ efflux and uptake in mitochondria following addition of PhAsO. The reaction medium and measurements are described under Materials and Methods. Succinate (2 mM) was added to 1 mg/ml rat liver mitochondria, and when pH was equilibrated 5 μM PhAsO was introduced. The other additions were 0.25 ng valinomycin/mg protein and 0.2 μg FCCP, as shown. HCl was added for calibration of the system.

presence of oligomycin (data not shown). If the reaction medium contains valinomycin, a K^+ ionophore, there is rapid extrusion of H^+ which is known to be caused by the uptake of the cation driven by the membrane potential (negative inside) (trace 2) and consequent further oxidation-driven H^+ extrusion. If PhAsO is added at this time, there is no decrease in the medium pH as in trace 1, but the subsequent discharge of the pH gradient still occurs. Thus, in the presence of valinomycin, the arsenical produces no further H^+ extrusion. The effects of valinomycin and the first phase of the PhAsO effect are operationally similar.

The effects of Cd^{2+} are similar to those of PhAsO (unpublished data), but more rapid at the optimal concentration with no detectable lag. Thus, the early H^+ extrusion is easier to study with the slower-acting PhAsO.

Figure 2 shows that the first phase of the PhAsO effect is potentiated by 2-mercaptoethanol, i.e., the maximum gradient is established sooner. In experiments where valinomycin was present and the H^+ extrusion was not rate limiting, the rate of the second phase (i.e., discharge of gradient) was accelerated in the presence of mercaptoethanol (data not shown). A 10-fold excess of the thiol produced the largest reduction in lag time as well as the maximum increase in the rate of discharge in the pH gradient. Further increase in thiol tended to slow the responses. The potentiating effect of the mercaptoethanol may be attributed to the greater hydrophobicity and

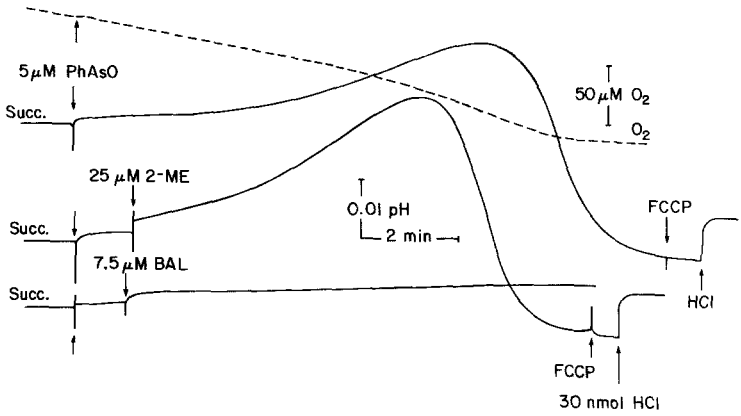

Fig. 2. Potentiation of arsenical effects by 2-mercaptoethanol and reversal by BAL. The experiment was as in Fig. 1 except for the addition of 25 μM 2-mercaptoethanol and 7.5 μM BAL as shown.

membrane permeability of the thioarsenical compared to that of PhAsO alone. The presence of BAL in much lower concentration, however, abolished both arsenical effects completely. Similar results have been obtained when Cd^{2+} was used in place of PhAsO (unpublished data).

Arsenite (0.1–1.0 mM) had no effect on the medium pH but in the presence of equimolar BAL produced the biphasic response. Thus, both reaction sites are inaccessible to the membrane-impermeable AsO_2^- but become available to the hydrophobic dithioarsenite ($Na–As\langle^{S}_{S}\rangle$). Higher levels of BAL (2- or 3-fold excess) completely prevented these pH changes.

Diamide (diazenedicarboxylic acid-bisdimethylamide) has been claimed to be a dithiol reagent (Stiggal et al., 1979). However, we have been unable to produce changes with diamide similar to those obtained wth Cd^{2+} or PhAsO.

During the first phase of the reaction (i.e., H^+ extrusion) there is significant increase in respiration. The second phase shows consistent 2- to

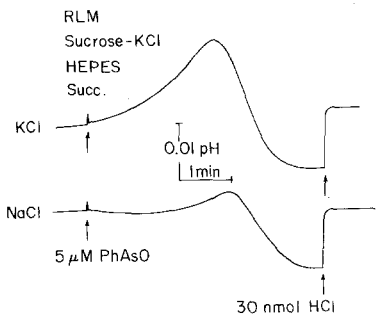

Fig. 3. Relative effects of K^+ and Na^+ on the H^+ efflux activated by PhAsO. The experiment was as in Fig. 1 except NaCl was used instead of KCl where shown.

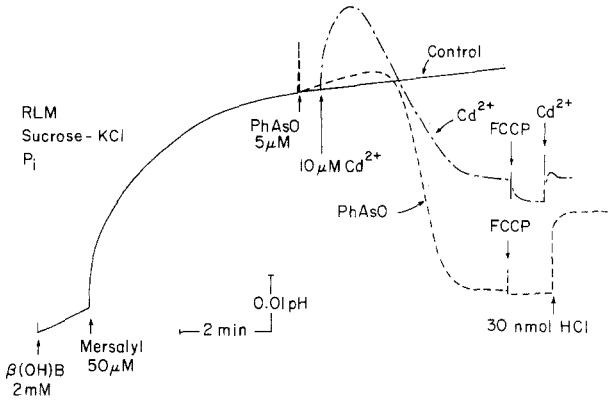

Fig. 4. Differential effects of Mesalyl compared to PhAsO and Cd^{2+} on discharge of the pH gradient. The experiment was as in Fig. 1 except for the use of 2 mM 3-hydroxybutyrate and 0.5 mM NAD^+ as substrate instead of succinate. The various additions were as shown.

3-fold increase in respiration, which is then followed at the end by lower respiration. In earlier work, we have shown that uncoupling is accompanied by extensive swelling of the mitochondria and possibly other secondary changes (Fluharty and Sanadi, 1962).

The medium for the experiments in Figs. 1 and 2 contained K^+. If K^+ is replaced by Na^+, the H^+ extrusion is much slower and its magnitude less at the time when the second, uncoupling phase sets in (Fig. 3).

In order to determine whether the H^+ extrusion and H^+ uptake effects (i.e., the first and second phase respectively) can be attributed to the same or to different sites of action, we have tested the effects of several other thiol-binding compounds. Mersalyl, i.e., *O*-[3-(hydroxymercuri)-2-methoxypropyl]carbamoylphenoxyacetic acid, and *p*-hydroxymercuribenzoate produced the first phase of the response or H^+ extrusion, but did not cause the subsequent decay of the pH gradient (Fig. 4). Addition of Cd^{2+} or PhAsO after the new steady state had been established, however, produced discharge of the H^+ gradient. The slight increase in pH gradient, which precedes the discharge, may be because these compounds appear to be more effective in activating H^+ extrusion than mersalyl and change the steady-state pH further before uncoupling sets in.

Discussion

The results clearly show that Cd^{2+} and PhAsO react with two different types of thiol groups in rat liver mitochondria. One site is K^+ dependent and

sensitive to *p*-hydroxymercuribenzoate and mersalyl, and is concerned with cation-linked H^+ ejection from respiring mitochondria (Fig. 4). It is distinct from the second or uncoupling site since these inhibitors do not discharge the pH gradient under the same conditions. Since mersalyl and *p*-hydroxymercuribenzoate are relatively membrane impermeable (Gaudemer and Latruffe, 1974), it would appear that the site reacting with them and responsible for the H^+ efflux is extrinsic and on the cytochrome *c* side of the inner membrane. The uncoupling site, on the other hand, appears to be in a hydrophobic environment, or at least a hydrophobic barrier has to be penetrated before it becomes available, as proposed earlier (Fluharty and Sanadi, 1961, 1962).

Another new aspect of this research is the finding that H^+ extrusion activated by Cd^{2+} and PhAsO is prevented by low levels of BAL but not by even a larger excess of 2-mercaptoethanol. Thus, this component also has a site which shows dithiol type of affinity for these compounds, in addition to being accessible to membrane impermeable monothiol reagents such as mersalyl. The results suggest that this dithiol-type group, in fact, may be extrinsic to the membrane.

Brierley (1976) has recently reviewed the extensive data on monovalent cation uptake (particularly K^+) and coupled H^+ extrusion from mitochondria. His laboratory has followed these ion distributions by monitoring mitochondrial swelling. In our earlier work, we have also observed mitochondrial swelling in the presence of Cd^{2+} or organic arsenical (Fluharty and Sanadi, 1962, 1963). The energy-dependent K^+ uptake is reported to be cryptic and activated by thiol inhibitors such as mersalyl and metals such as Zn^{2+}, Cd^{2+}, Cu^{2+}, and Pb^{2+}. Among the models that have been considered for the uptake are (a) electrophoretic distribution of cations in response to the electrical negative charge inside respiring mitochondria (Mitchell, 1966; Brierley, 1974), (b) cation/H^+ exchange on the same carrier (Azzone and Massari, 1973; Diwan *et al.*, 1977 Garlid, 1979), and (c) transport by ionophoric proteins (Harris and Pressmen, 1969). Currently available data do not permit a choice between these mechanisms. PhAsO and Cd^{2+} are more selective reagents (particularly in the presence of 2-mercaptoethanol), and offer a good chance to study the mechanisms of monovalent cation transport in mitochondria.

Acknowledgements

The authors appreciate the competent assistance of Radha Narayana in this work. This work was supported by grants from the National Institutes of Health (ES02167, GM26764, and GM13641). J. B. Hughes was supported by an NIH postdoctoral traineeship (HL07266).

References

Azzone, G. F., and Massari S. (1973). *Biochim. Biophys. Acta* **301,** 195–226.
Brierley, G. P. (1976). *Mol. Cell. Biochem.* **10,** 41–62.
Brierley, G. P. (1974). *Ann. N.Y. Acad. Sci.* **227,** 398–411.
Diwan, J. J., Markoff, M., and Lehrer, P. H. (1977). *Indian J. Biochem. Biophys.* **14,** 342–346.
Fluharty, A. L., and Sanadi, D. R. (1961). *Proc. Natl. Acad. Sci. USA* **46,** 608–616.
Fluharty, A. L., and Sanadi, D. R. (1962). *Biochemistry* **1,** 276–281.
Fluharty, A. L., and Sanadi, D. R. (1963). *Biochemistry* **2,** 519–522.
Garlid, K. D. (1979). *Biochem. Biophys. Res. Commun.* **87,** 842–846.
Gaudemer, Y., and Latruffe, P. L. (1974). *FEBS Lett.* **54,** 30–34.
Harris, E. J., and Pressman, B. C. (1969). *Biochim. Biophys. Acta* **172,** 66–70.
Jacobs, E. E., Jacob, M., Sanadi, D. R., and Bradley, L. B. (1956). *J. Biol. Chem.* **223,** 147–156.
Johnson, D., and Lardy, H. (1967). *Methods Enzymol.* **10,** 94–95.
Mitchell, P. (1966.) *Biol. Rev.* **41,** 445–502.
Stiggal, D. L., Galante, Y. M., Kiehl, R., and Hatefi, Y. (1979). *Arch. Biochem. Biophys.* **196,** 638–644.