Journal of Bioenergetics and Biomembranes, Vol. 28, No. 6, 1996

Effectors of the Mammalian Plasma Membrane NADH-Oxidoreductase System. Short-Chain Ubiquinone Analogues as Potent Stimulators

François Vaillant,[1] Jari A. Larm,[1] Gabrielle L. McMullen,[1,2] Ernst J. Wolvetang,[1,3] and Alfons Lawen[1]

Received October 30, 1995; accepted April 22, 1996

In the presence of effectors variations in the two recognized activities of the plasma membrane NADH-oxidoreductase system were studied in separate, specific *in vitro* assays. We report here that ubiquinone analogues that contain a short, less hydrophobic side chain than coenzyme Q-10 dramatically stimulate the NADH-oxidase activity of isolated rat liver plasma membranes whereas they show no effect on the reductase activity of isolated membranes. If measured in assays of the NADH:ferricyanide reductase of living cultured cells these compounds have only a limited effect; the oxidase activity of whole cells is not measurable in our hands. We have furthermore identified selective inhibitors of both enzyme activities. In particular, the NADH-oxidase activity can be significantly inhibited by structural analogues of ubiquinone, such as capsaicin and resiniferatoxin. The NADH:ferricyanide reductase, on the other hand, is particularly sensitive to pCMBS, indicating the presence of a sulfhydryl group or groups at its active site. The identification of these specific effectors of the different enzyme activities of the PMOR yields further insights into the function of this system.

KEY WORDS: Plasma membrane NADH-oxidoreductase; NADH-oxidase; NADH:ferricyanide reductase; ubiquinone analogues; ρ^0 cells.

INTRODUCTION

All eukaryotic cells studied so far possess a redox system located at the plasma membrane level and characterized as plasma membrane NADH-oxidoreductase[4] (PMOR; Crane *et al.*, 1985, 1991; Morré and Brightman, 1991). A definitive role in cellular signalling has been attributed to this enzyme system (Crane *et al.*, 1991; Sun *et al.*, 1992b) as well as a possible involvement in transferrin-independent iron uptake by cells (Inman and Wessling-Resnick, 1993). We have also recently demonstrated that up-regulation of the PMOR activity is a prerequisite for the viability and growth of ρ^0 cells (cells devoid of mitochondrial (mt) DNA) (Larm *et al.*, 1994) and ρ^d cells (cells with only a very low copy number of mtDNA; Vaillant and Nagley, 1995 and Vaillant, manuscript in preparation). The PMOR is proposed to re-oxidize excess cytosolic NADH to NAD^+, transferring electrons from inside the cell to extracellular electron acceptors, thereby maintaining an appropriate NAD^+/NADH ratio (Lawen *et al.*, 1994). The natural electron acceptor of the reductase activity has yet to be identified, but

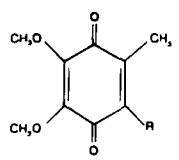
[1] Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3168, Australia.
[2] Present address: Australian Catholic University, Aquinas Campus, Ballarat VIC 3350, Australia.
[3] Present address: Institute for Reproduction and Development, Monash Medical Centre, 266 Clayton Road, Clayton VIC 3168, Australia.
[4] Abbreviations used: DCIP, 2,6-dichlorophenol-indophenol; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); FAC, ferric ammonium citrate; pCMBS *p*-chloromercuriphenylsulfonic acid; PMOR, plasma membrane NADH-oxidoreductase; coenzyme Q-0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; SH, sulfhydryl group.

0145-479X/96/1200-0531$09.50/0

transferrin has been proposed to be a likely candidate (Löw *et al.*, 1987). Ferricyanide, a nonphysiological electron acceptor, has been used to establish the presence of a NADH:ferricyanide reductase activity in the PMOR system (Clark *et al.*, 1981; Sun *et al.*, 1984a). 2,6-Dichlorophenol-indophenol (DCIP) can also be used as an artificial electron acceptor (Goldenberg *et al.*, 1979). Other recognized *in vitro* electron acceptors that are present naturally in biological systems include ubiquinone (Villalba *et al.*, 1995) and ascorbate (Navas *et al.*, 1988). Interestingly, ferricyanide or DCIP, when used instead of pyruvate, are each able to maintain the viability of ρ^0 cells (Martinus *et al.*, 1993; Larm *et al.*, 1994) which are usually grown in medium supplemented with pyruvate and uridine (Desjardins *et al.*, 1985; King and Attardi, 1989; Vaillant *et al.*, 1991). Ferricyanide and DCIP are not permeant to plasma membranes and are therefore considered to maintain viability of these cells by acting at the level of the PMOR.

The PMOR system is a multienzyme complex exerting at least a second enzymatic activity, namely an NADH-oxidase (Brightman *et al.*, 1992; Morré and Brightman, 1991). Here, the re-oxidation of cellular NADH is coupled to the reduction of O_2 to water. Each of the two redox activities is likely to be associated with a different polypeptide or polypeptides but a functional linkage between both has been proposed. The PMOR system can be stimulated by a number of growth factors, cytokines, and hormones (Crane *et al.*, 1985), but the exact nature of the growth stimulation signal(s) generated by the PMOR system, in response to extracellular signals, is still unknown. However, an important observation is that the PMOR is also responsive to effectors other than growth factors and hormones *sensu strictu*. Ubiquinone, an ubiquitous molecule in cellular metabolism (Crane *et al.*, 1993), has previously been shown to stimulate the NADH-oxidase activity of isolated rat plasma membranes (Brightman *et al.*, 1992) and to be an essential component of the PMOR system (Sun *et al.*, 1992b). Inhibition of the oxidase activity by ubiquinone-related compounds such as capsaicin, on the other hand, has been shown to readily induce cell death by apoptosis (Lawen *et al.*, 1994; Wolvetang *et al.*, 1996). Ubiquinone, also known as coenzyme Q-10, has a long side chain composed of 10 isoprenoid units at position 6 of the 2,3-dimethoxy-5-methyl-1,4-benzoquinone (coenzyme Q-0) (see Table I). This side chain is responsible for the highly hydrophobic character of the molecule. In this report, we have utilized ubiquinone analogues with shorter side chains (and which are therefore less hydrophobic than ubiquinone) to further investigate the role of ubiquinone and its analogues for the activity of the enzyme. In a previous study, these analogues have been shown to stimulate the activity of the NADH:ferricyanide reductase of whole cells (Larm *et al.*, 1995). In order to differentiate the two redox

Table I. Structures and Hydrophobicities of Ubiquinone Analogues



R=H: coenzyme Q-0[a]

Compound	R (C_6 substituent)	Hydrophobicity (log P)
Ubiquinone	$-(CH_2-CH=C(CH_3)-CH_2)_{10}-H$	>10
Q_{3C}	$-CH_2-CH=CH_2$	1.3
Q_{4C}	$-CH_2-CH=CH-CH_3$ and $-CH(CH_3)-CH=CH_2$	2.0
Q_{5C}	$-(CH_2)_4-CH_3$	3.5
Q_{6C}	$-CH_2-CH=CH-CH=CH-CH_3$	4.2
Q-2	$-CH_2-CH=C(CH_3)-CH_2-CH_2-CH=C(CH_3)-CH_3$	5.1
Q_{9C}	$-(CH_2)_8-CH_3$	7.3
Q_{10C}	$-(CH_2)_9-CH_3$	8.04
Q_{11C}	$-(CH_2)_{10}-CH_3$	8.75

[a] Coenzyme Q-0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; Q_{3C}, prop-2'-enyl-coenzyme Q-0; Q_{4C}, but-2'-enyl-coenzyme Q-0 and 1'-methyl-prop-2'-enyl-coenzyme Q-0; Q_{5C}, pentyl-coenzyme Q-0; Q_{6C}, hexa-2',4'-dienyl-coenzyme Q-0; Q-2,3',7'-dimethyl-octa-2',6'-dienyl-coenzyme Q-0; Q_{9C}, nonyl-coenzyme Q-0; Q_{10C}, decyl-coenzyme Q-0 Q_{11C}, undecyl-coenzyme Q-0

activities of the PMOR and better define their constituent(s), we have searched for specific effectors of both, the NADH-oxidase and NADH:ferricyanide reductase activity. We were able to distinguish the two activities on the basis of their differential responses to the stimulators or inhibitors examined.

MATERIALS AND METHODS

Synthesis and Source of Ubiquinone Analogues and Other Chemicals

Ubiquinone analogues were synthesized by addition of a substituent at position 6 of coenzyme Q-0. The alkyl ubiquinone analogues (saturated side chain) were synthesized according to Wan *et al.* (1975). Ratios of 1.5–2 molar equivalents of diacyl peroxide with respect to coenzyme Q-0 resulted in best yields (40–50% analogues). The unsaturated analogue 2′-propenyl-Q was synthesized from coenzyme Q-0 and allylated tributyltin (Naruta, 1980). The allylated tributyltin was obtained by sonication of 3-chloro-1-propene. The analogs were purified by flash chromatography on silica gel 60 (Merck, Kilsyth, Australia) using 10% ethyl acetate/light petroleum ether. Characterization was carried out by means of ^{1}H and ^{13}C NMR spectra (Bruker 200 MHz instrument), infrared (Perkin Elmer 1600 Series FTIR), ultraviolet/visible (Beckman DU7000 spectrophotometer), and mass (VG Trio GC-MS) spectra.

The isoprenoid analogues Q-1 and Q-2 were generous gifts from Eisai Co., Tokyo, Japan. The undecyl analogue was a kind gift of Dr. E. Berry, University of California, Berkeley, USA. Decylubiquinone was purchased from Sigma Chemical Co., Castle Hill, Australia.

The quinones were prepared and stored as ethanolic solutions and the quinone concentration of the individual stock solutions were determined by absorption at 275–280 nm (ε = 14.5 $mM^{-1}cm^{-1}$). The partition coefficients in cyclohexane/water were evaluated using parameters previously reported (Braun *et al.*, 1986).

Capsaicin, dihydrocapsaicin, adriamycin, and ouabain were obtained from Sigma Chemical Co. Resiniferatoxin was obtained from Fluka BioChemicals, Switzerland. Rotenone was purchased from BDH, Kilsyth, Australia. *N*-nonyl-4-aminoquinozaline (SAN 547A) was a kind gift of Sandoz Agro Ltd. (Basel, Switzerland).

Cell Line and Culture Conditions

The human lymphoblastoid cell line Namalwa (Nyormoi *et al.*, 1973), and derived strains were cultured as described previously (Vaillant and Nagley, 1995) in RPMI-1640 medium (Irvine Scientific Cytosystems, Melbourne, Australia) complemented with 10 mM HEPES, pH 7.4, 100 IU/ml penicillin, 100 μg/ml streptomycin, and finally supplemented with 10% heat-inactivated fetal calf serum (Commonwealth Serum Laboratories, Parkville, Australia). The Namalwa ρ^0 cell line was obtained as described (Vaillant and Nagley, 1995) by propagating ρ^+ cells in medium containing 50 ng/ml ethidium bromide, 1 mM pyruvate, and 50 μg/ml uridine for more than 16 weeks. Cells were washed free of ethidium bromide and passaged in medium containing pyruvate and uridine. The ρ^0 cell population was subcloned (Vaillant and Nagley, 1995) and subclones, derived from one single progenitor cell, were isolated. A particular clone designated clone 12 was used in this study (referred to here as ρ^0 cells).

Preparation of Plasma Membrane from Rat Liver and Purity Assays

Plasma membranes were prepared from male Wistar rat livers (Navas *et al.*, 1989). Rats, fed *ad libitum*, were fasted 24 hours before being decapitated. The livers were removed and weighed. Approximately 15–20 g of liver were immediately diced and washed in ice-cold 0.25 M sucrose and 5 mM Tris-HCl buffer (pH 7.2); further steps were carried out on ice. The homogenate was centrifuged at 1,000 *g* for 10 min and the supernatant, free of nuclei and unbroken cells, was further centrifuged at 28,000 *g* for 30 min. The supernatant was carefully removed without disturbing the mitochondrial pellet. This supernatant was centrifuged at 105,000 *g* for 60 min. The pellet was resuspended in a small volume of 1 mM sodium bicarbonate (pH 7.4) and the plasma membrane fraction was purified by an aqueous two-phase separation method as described in Morré and Morré (1989). The purity of the plasma membrane fraction was assessed using marker enzyme activity assays specific for the various subcellular fractions as follows.

K^+-stimulated, ouabain-inhibited, ATPase activity (plasma membrane fraction) was determined using an adaptation of a previously described method (Navas *et al.* 1988). The reaction mixture contained 10 mM

imidazole buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM *p*-nitrophenylphosphate (Tris salt), 200–250 μg of protein, and either 10 mM KCl or 100 μM ouabain. The reaction was carried out at 37°C for 15 min and stopped by addition of 2 ml 0.5 M NaOH. The absorbance at 410 nm was measured and the enzyme activity was determined using the difference in absorbance between the substrate plus KCl (stimulated enzyme) and substrate plus ouabain (inhibited enzyme).

Succinate-cytochrome *c* reductase activity (mitochondria) was determined by adapting a literature method (Sottocasa *et al.*, 1967) where the 1 ml assay mixture contained 20 μM oxidized cytochrome *c* (dissolved in 20 mM Tris-HCl, pH 7.4, 2 mM EDTA), 20 mM sodium succinate, and 1 mM KCN in 25 mM potassium phosphate buffer, pH 7.5. The reaction was started by the addition of 10–20 μg protein and the reduction of cytochrome *c* was followed spectrophotometrically at 550 nm (ε = 18.7 mM^{-1} cm^{-1}) at room temperature for 20 min.

NADPH-cytochrome *c* reductase activity (membrane-bound endoplasmic reticulum) was determined by a modification of the method of Mahler (1955). The 1-ml incubation mixture containing 20 μM oxidized cytochrome *c* (dissolved as above) and 10–20 μg protein in 25 mM potassium phosphate buffer, pH 7.5, was preincubated with 1 mM rotenone for 10 min at room temperature. The reaction was started by addition of 150 μM NADPH and the reduction of cytochrome *c* was followed as above.

Esterase activity (soluble endoplasmic reticulum) was determined by a modification of a previously described method (Beaufay *et al.*, 1974). The 1-ml reaction mixture contained 20 mM potassium phosphate buffer, pH 6.6, 1 mM EDTA, 0.1% (w/v) Triton X-100, and 10–20 μg protein. The reaction was started by the addition of 3 mM *p*-nitrophenyl acetate dissolved in ice-cold methanol. The production of *p*-nitrophenol was measured spectrophotometrically at 405 nm at 37°C.

β-hexosaminidase activity (lysosomes) was determined as previously described (Wanders *et al.*, 1984).

Lactate dehydrogenase activity (cytosol) was determined by a modification of a previously described method (Wróblewski and LaDue, 1955). The 1-ml reaction mixture contained 25 mM potassium phosphate buffer, pH 7.4, 1 mM KCN, 50 μM NADH, and 10–20 μg protein. The reaction was started by the addition of 100 μM pyruvate and the decrease in the absorbance of NADH was followed spectrophotometrically at 340 nm at room temperature for 15 min.

Protein concentrations were determined using Bradford reagent (Bradford, 1976) according to the manufacturer's instructions (BioRad, North Ryde, Australia).

Plasma Membrane Oxidoreductase Activities

Activity of the NADH:ferricyanide reductase was measured in intact cells, adapting a method described previously (Crane *et al.*, 1982). Briefly, cells were washed in phosphate-buffered saline (0.15 M sodium chloride, 20 mM sodium phosphate, pH 7.2). 0.5–1.0 × 10^6 cells were resuspended in 1 ml phosphate-buffered saline containing 10 mM glucose, 1 mM pyruvate, and 2 μM rotenone. Following preincubation for 10 min at 37°C, the reaction was initiated by the addition of 250 μM potassium ferricyanide (final concentration) and monitored with an Aminco DW2a spectrophotometer at 420/500 nm for 4 min at 37°C. The NADH:DCIP reductase activity in intact cells was measured similarly, but 20 μM DCIP was used as substrate and its reduction was followed at 600/700 nm. Molar extinction coefficients of 1,000 and 21,000 $M^{-1}cm^{-1}$ were used for ferricyanide and DCIP, respectively.

The reductase activity (NADH:ferricyanide or NADH:DCIP reductase) of isolated plasma membranes was determined as follows. Plasma membrane fractions (30–40 μg of protein, preincubated for 10 min with 2 μM rotenone) were added to a 1-ml reaction mixture containing 50 μM NADH, 1 mM KCN, and 250 μM potassium ferricyanide (or 20 μM DCIP) in 40 mM Tris-MES buffer, pH 7.4. The reduction of substrate was followed as above. The NADH:ferric ammonium citrate reductase activity was measured as previously described (Bérczi *et al.*, 1991). Bathophenanthroline disulfonic acid (100 μM), ferric ammonium citrate (80 μg/ml corresponding to approximatively 14 μg/ml of ferric iron), and NADH (50 μM) were added first to the reaction cuvette. Once a basal rate had been recorded, the plasma membrane preparation was added to the reaction mixture.

NADH-oxidase activity of isolated plasma membranes was determined as previously described (Brightman *et al.*, 1992) except that the plasma membrane preparation was preincubated in 2 μM rotenone for 10 min. The reaction was started by the addition of 130 μg of protein.

In all cases, effectors were added to the reaction mixture after a basal rate had been established, but inhibitors were preincubated with the plasma membrane preparation or the cell suspension for 10 min.

Superoxide Formation

Superoxide formation was determined in a Luminometer (LKB Wallac) using the chemiluminescence probe L-012 (Nishinaka *et al.*, 1993) as previously described (Larm *et al.*, 1995). Plasma membrane fractions were incubated in 0.25 ml of 40 mM Tris/MES buffer, pH 7.0, containing 0.5 mM L-012 and 150 μM NADH and the specific effectors under study.

RESULTS

We have synthesized a number of ubiquinone analogues that have a shortened side chain at position 6 of the benzoquinone ring and, consequently, have a generally lower hydrophobicity coefficient than ubiquinone (Table I). Hypothetically, these compounds would therefore be more readily accessible to the various sites of cellular membrane-bound enzyme activity. It was further reasoned that the less hydrophobic characteristics of these ubiquinone analogues would modify their pharmacokinetic properties and increase their general availability to the cells. Indeed, these analogues successfully sustained the viability and growth of Namalwa ρ^0 cells in the absence of pyruvate at a concentration of 1 μM (Lawen *et al.*, 1994) compared to the 10 μM of ubiquinone needed for the same effect (Martinus *et al.*, 1993). To elucidate the mechanism by which these compounds support the growth of ρ^0 cells, we analyzed their effects on various enzymic activities of the PMOR system. We had already demonstrated that the NADH:ferricyanide reductase activity of Namalwa ρ^+ cells is slightly stimulated by a factor of about 1.8 following the addition of 1 μM of Q_{3C} (Larm *et al.*, 1995). Similar stimulation can also be observed using other analogues such as Q_{4C} or Q_{5C} (data not shown). The NADH:ferricyanide reductase of Namalwa ρ^0 cells, although already up-regulated about 4-fold when compared to Namalwa ρ^+ cells (Larm *et al.*, 1994), can be further stimulated 1.2- to 2.2-fold by the addition of micromolar concentrations of the ubiquinone analogues (Fig. 1).

To further characterize the two individual activities of the PMOR system, we have isolated the plasma membrane fraction from rat livers. The purity of the plasma membrane preparations was assessed on the basis of marker enzyme assays specific for the subcellular fractions (see Materials and Methods). Only plasma membrane preparations with a purity of at least 90% were selected for further use. Contaminations with other subcellular fractions were as follows: ~1.5% mitochondria, ~5% endoplasmic reticulum, ~2% lysosomes. Surprisingly, the NADH:ferricyanide reductase activity of these rat liver plasma membrane preparations was not stimulated by the various ubiquinone analogues. This result is obviously in disagreement with the observations made with whole cell NADH:ferricyanide reductase activity. Besides possible but questionable structural and/or functional differences between enzymes originating from two closely related mammalian systems (rat plasma membrane and human lymphoblastoid cells), one possible explanation for this result is the loss of an essential component of the PMOR system during the isolation procedure. One or more loosely associated cofactor(s) could be essential for the stimulation of the system by the ubiquinone analogues, although not for the basal NADH:ferricyanide reductase activity. We tested whether this cofactor could be ubiquinone or a ferric salt. However, inclusion of either 10 μM ubiquinone or 1 μM ferric chloride together with individual analogues had no stimulatory effect, the NADH:ferricyanide activity remaining similar to that of the control samples (data not shown).

The activity of the plasma membrane NADH-oxidase represents less than 10% of the overall cellular oxygen consumption and was therefore not detectable in our hands when assayed in viable cells. To detect the NADH-oxidase activity, it is necessary to use isolated rat liver plasma membrane fractions where oxidase measurements can be made (Morré and Brightman, 1991) without interference from other oxygen-consuming activities. All short-chain ubiquinone analogues showed a strong stimulation of the NADH-oxidase activity (Fig. 1). Addition of 50 μM of Q_{3C} stimulated the oxidase activity by a factor of 10. In comparison, only a 2.2-fold stimulation was observed with the same amount of Q_{11C}. Increasing the concentration of Q_{11C} did not further increase the degree of stimulation. An indirect relationship between the hydrophobicity coefficient and the degree of stimulation was observed (Fig. 1). In effect, the lower the hydrophobicity coefficient was, the higher the degree of stimulation. Only Q_{6C} stimulates the oxidase to a somewhat higher degree than would be expected from that relationship. The basis for this is not known yet

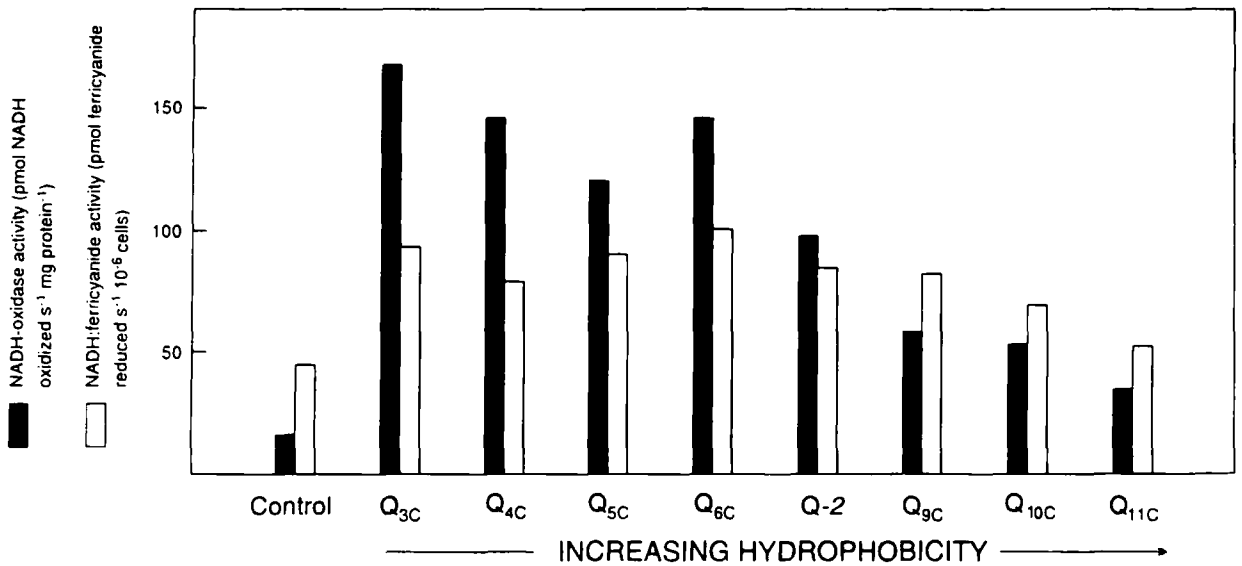


Fig. 1. Stimulation of PMOR activities by ubiquinone analogues. The NADH:ferricyanide activity of ρ^0 cells (empty bars) and the NADH-oxidase activity of isolated rat liver plasma membrane (filled bars) were measured as described in Materials and Methods. Ubiquinone analogues (20 μM for the NADH:ferricyanide activity and 50 μM for the NADH-oxidase activity) were added to the reaction mixture after a basal rate had been established. For hydrophobicity coefficients, refer to Table I.

but the structure of the side chain does not present any particularly distinct feature when compared with the other analogues.

Increase of the NADH consumption in the NADH-oxidase assay in the presence of ubiquinone analogues may be explained by the stimulation of the activity of another oxidoreductase than the PMOR. A possible contamination of our preparations with DT-diaphorase (NADPH:quinone reductase; E.C. 1.6.99.2.) can be discounted on the basis of the very weak reduction of NADPH by isolated rat plasma membrane, in both the NADH-oxidase and the NADH:ferricyanide reductase assays, even in the presence of ubiquinone analogues (data not shown). However, an artificial oxidase system or "futile cycle," reoxidizing NADH and using O_2 as a final electron acceptor with a quinone/semiquinone intermediate, could be set up in the presence of the ubiquinone analogues (Hochstein, 1983). In this case a significant production of superoxide would be expected. Menadione, for example, is known to be able to create artificial oxidase activities with cellular dehydrogenases, leading to the production of superoxide from the semiquinone form (Afanas'ev *et al.*, 1990). We have already reported that, in whole cells, the stimulation of the NADH:ferricyanide reductase is not correlated with an increase superoxide production (Larm *et al.*, 1995). Using the chemiluminescent probe L-012, we have also not been able to detect any significant superoxide production by isolated plasma membrane upon stimulation with the ubiquinone analogues or menadione. Furthermore, the NADH-oxidase activity is inhibited by the specific inhibitors examined in this report to a similar extent irrespective of whether it is stimulated or not by ubiquinone analogues. Taken together, these results strongly suggest that the ubiquinone analogues do actually stimulate the plasma membrane NADH:oxidase activity and that the increased NADH consumption is not artificial.

A number of inhibitors of the PMOR system have been previously reported, such as capsaicin, chloroquine, and retinoic acid (Sun *et al.*, 1984b, 1987, 1992b, 1995; Brightman *et al.*, 1992). We have investigated the inhibitory effect of a range of compounds on the NADH-ferricyanide reductase and NADH-oxidase in whole cells and isolated plasma membranes (Table II). In particular, we focussed our attention on compounds that are structurally related to ubiquinone. The vanilloid capsaicin moderately inhibited the NADH-oxidase activity at a concentration of 200 μM. Dihydrocapsaicin and resiniferatoxin, which are analogues of capsaicin, were found to be more potent inhibitors of the NADH-oxidase activity. More than 80% inhibition was achieved with 200 μM dihydrocapsaicin or 20 μM resiniferatoxin. The latter is the most potent oxidase inhibitor of the three vanilloids.

Table II. Effect of Inhibitors on the Activities of the PMOR

	Isolated rat liver plasma membrane			Namalwa ρ^+ cells
	NADH-oxidase	NADH:ferric salt reductase	NADH:DCIP reductase	NADH:ferricyanide reductase
Control activity	14.6 ± 0.17 pmol·sec^{-1}·mg protein^{-1}	10.1 ± 0.4[c] pmol·sec^{-1}·mg protein^{-1}	285 ± 9 pmol·sec^{-1}·mg protein^{-1}	10.5 ± 0.5 pmol·sec^{-1}·10^{-6} cells
Inhibitor[a]	% inhibition relative to untreated control			
Resiniferatoxin (10 μM)	98 ± 2	0[c]	0	0
Dihydrocapsaicin (200 μM)	80 ± 6	0[d]	0	n.m.[b]
Capsaicin (200 μM)	50 ± 4	0[d]	0	n.m.
Adriamycin (2 μM)	52 ± 5	0[d]	0	n.m.
SAN547A (300 μM)	41 ± 4	0[c,d]	0	0
pCMBS (20 μM)	0	94 ± 1[e]	98±2	88 ± 5[e]

[a] The concentration at which maximum inhibition is observed indicated in parentheses.
[b] n.m. indicates that the reaction was not measurable due to nonspecific reaction of the inhibitor with the assay substrate.
[c] The reaction was measured using ferricyanide as ferric salt.
[d] The reaction was measured using ferric ammonium citrate as ferric salt.
[e] The effective concentration was 100 μM.

The activated NADH-oxidase is inhibited to a similar extent; 20 μM resiniferatoxin, for example, inhibited Q_{3C}-stimulated activity by 72%. *N*-nonyl-4-amino-quinozaline (SAN 547A), an extremely potent inhibitor of mitochondrial complex I (total inhibition at 20 μM concentration), reduced the NADH oxidation by only about 40% at a concentration of 300 μM. We observed a 54% inhibition of the NADH-oxidase in isolated rat liver plasma membranes after addition of 2 μM adriamycin (doxorubicin). Adriamycin, capsaicin, and dihydrocapsaicin showed enzyme-independent reduction of ferricyanide when included in the NADH:ferricyanide reductase assay mixture. This assay was therefore not suitable to assess the effect of these compounds on the NADH:ferricyanide reductase activity. We have used two alternative assays to investigate these compounds. Ferricyanide was first replaced by another ferric salt, namely ferric ammonium citrate (FAC), according to a procedure already described (Bérczi *et al.*, 1991). DCIP, as an alternative electron acceptor, was used to measure the reductase activity (NADH:DCIP reductase activity in this case). While it is known that the NADH:ferric salt reductase can be measured equivalently with ferricyanide or FAC (Löw *et al.*, 1987), the identity between the NADH:ferricyanide reductase and the NADH:DCIP reductase has not been formally established. FAC and DCIP do not present any enzyme-independent reaction with any of the inhibitors used in this study. Using these systems, we found that concentrations of capsaicin, dihydrocapsaicin, or adriamycin that were inhibitory to the NADH-oxidase (50% inhibition) had no effect on the reductase activity of isolated plasma membranes (Table II). Similarly, resiniferatoxin, which can be included in the incubation mixture of the NADH:ferricyanide reductase assay, had no inhibitory effect on the reductase activity (Table II). We were, however, unable to obtain reproducible rates for the NADH:FAC reductase activity of viable cells. In this case, only the NADH:DCIP reductase activity was measured in the presence of the inhibitors.

p-chloromercuriphenylsulfonic acid (pCMBS), which reacts specifically with sulfhydryl (SH) groups, proved to be a potent inhibitor of the reductase activities (NADH:ferricyanide and NADH:DCIP reductases), both in whole cells and isolated plasma membranes. A concentration of 20 μM pCMBS totally inhibited the NADH:ferricyanide reductase activity of isolated plasma membranes; a higher concentration was required for the inhibition of whole cell reductase activity (Table II). In the latter case the extent of inhibition varied according to the acceptor used (88 ± 5% for ferricyanide and 51 ± 6% for DCIP). The basis for this difference is not yet known. However, pCMBS had no effect on the NADH-oxidase activity of isolated rat liver plasma membrane. 5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB), another SH group reagent, had no effect on the reductase activity. Obviously, pCMBS reacts with SH groups that are partially masked and inaccessible to DTNB. The site of action of pCMBS

is very likely to be an external –SH group since this compound penetrates the membranes only at a very slow rate (Aledort *et al.*, 1968; VanSteveninck *et al.*, 1965). As previously shown, pCMBS induces cell death at concentrations of 100 μM and above as measured by trypan blue exclusion (Larm *et al.*, 1994). In order to ascertain that an SH group is involved, cells were incubated with pCMBS for 10 min, and thereafter incubated with dithioerythreitol (DTE) for a further 10 min. Since DTE undergoes an enzyme-independent reaction with ferricyanide or DCIP, the cells were washed extensively prior to measurement of the enzyme activity. The addition of DTE almost totally reversed the pCMBS inhibition (Table III). DTE is unlikely to stimulate unspecifically the activity, since it has no effect on the uninhibited reductase (Table III). To further prove that the recovery of the activity was the result of the restoration of the –SH group(s) by DTE and not an unspecific effect of DTE, *N*-ethymaleimide (NEM) was used in a similar experiment. NEM also inhibits the NADH:ferricyanide activity (Table III) but binds in an irreversible manner to membrane sulfhydryl groups (van Iwaarden *et al.*, 1992). It was therefore unlikely that a restoration of the –SH group(s) by DTE would be observed, a reversion of the activity indicating here an unspecific effect. Indeed, after treatment with NEM, DTE did not restore any activity. Taken together, these results demonstrate that pCMBS reacts with a partially masked SH group, probably essential to the reductase activity, and that the reversion observed in the presence of DTE results from the restoration of this SH group.

DISCUSSION

The PMOR system illustrates a fundamental principle in the overall redox balance of mammalian cells. Up-regulation of the NADH:ferricyanide activity is a prerequisite for the viability of ρ^0 cells, suggesting that the PMOR may act as a backup or compensatory mechanism when mitochondrial oxidative phosphorylation is impaired (Larm *et al.*, 1994, 1995). Understanding variations of the activity of the PMOR in response to environmental changes or cellular alterations throws further light on the physiological role of this system. In this report, we have investigated the function of the PMOR system using positive and negative effectors of its constitutive activities.

The NADH:ferricyanide activity of ρ^0 cells that is already up-regulated as a result of the loss of mitochondrial DNA (Larm *et al.*, 1994) can be further stimulated (approximately 2-fold) by the addition of various short-chain analogues of ubiquinone. In addition, the NADH-oxidase activity of the plasma membrane can be dramatically increased upon addition of the same analogues (2- to 10-fold stimulation, Fig. 1). This is particularly significant in the case of Q-2 since this quinone is a very poor substrate of the mitochondrial NADH-oxidase (Lenaz *et al.*, 1975). As plasma membrane has not yet been isolated from Namalwa cells at high purity, this increase was only observed with rat liver preparations. It is also significant that the most potent inhibitors (capsaicin, dihydrocapsaicin, and resiniferatoxin) are structurally related to ubiquinone (benzoquinone ring substituted in position 6

Table III. Reversion of pCMBS inhibition by DTE[a]

	Control	1 mM DTE	100 μM pCMBS	100 μM pCMBS + 1 mM DTE	1 mM NEM	1 mM NEM + 1mM DTE
Nawalma ρ^+ cells,[b] pmol·sec^{-1}·10^{-5} cells	64.6 ± 2.7	61.4 ± 1.3	32.2 ± 1.6	58.8 ± 2.7	7.5 ± 0.8	8.95 ± 1.7
(percent of control)	(100)	(95)	(50)	(91)	(11)	(14)
Plasma membrane,[c] pmol·sec^{-1}·mg prot^{-1}	2.58 ± 0.27	(100)[d]	0.49 ± 0.06	1.23 ± 0.13	n.d.	n.d.
(percent of control)	(100)		(18)	(48)		

[a] Cells in PBS were incubated for 10 min at 37°C in the presence of pCMBS, NEM, DTE, or without additives. The cell suspension was then supplemented, as indicated with 1 mM DTE, and further incubated for 10 min. After washing the cell pellets at least twice with PBS the NADH:DCIP reductase activity was measured. In the case of plasma membrane, following incubation with the different compounds, the samples were dialyzed overnight against 1 mM sodium bicarbonate before assaying the NADH:ferricyanide reductase activity.

[b] NADH:DCIP reductase activity of whole cells, expressed as pmol DCIP reduced per sec per 10^5 cells.

[c] NADH:ferricyanide reductase activity of isolated rat liver plasma membrane expressed as pmol ferricyanide reduced per sec per mg of protein.

[d] In a separate experiment, it was shown that addition of DTE only to membranes and further dialysis did not significantly affect the NADH:ferricyanide reductase activity of isolated rat liver plasma membrane. However, as variations are observed in the final activity following dialysis, the numerical results are not displayed here.

with a long hydrophobic side chain). Ubiquinone is an essential component of the PMOR (Crane *et al.*, 1993) and its removal results in the loss of activity (Sun *et al.*, 1992b). Ubiquinone is most likely anchored in the lipid bilayer of the PMOR where it facilitates the electron flow between cytosolic NADH and external acceptors, probably establishing a functional link between the NADH:ferricyanide reductase and NADH-oxidase activities of the PMOR (Morré and Brightman, 1991; Crane *et al.*, 1991). A redox cycle may be proposed for the plasma membrane similar to the one that exists in the *bc*, complex of mitochondria and bacteria (Brandt and Trumpower, 1994). Proton expulsion from the cell during plasma membrane electron transport (Sun *et al.*, 1984a) is consistent with the feature of the Q cycle (i.e., transmembrane proton transport). It is not yet certain if the analogues used in this study act at the same site as ubiquinone as additional, less hydrophobic sites may exist in the PMOR system at which these analogues could interact. At these sites, the analogues would facilitate a much enhanced electron flow from NADH to high-reduction-potential electron acceptors. Insofar as such sites would be more accessible to hydrophilic ubiquinone-related compounds, this would explain the direct relationship between hydrophobicity coefficient and percentage of stimulation that we observed (Fig. 1).

The stimulation of the NADH:ferricyanide reductase activity was initially observed during the generation of ρ^0 cells from ρ^+ cells (Larm *et al.*, 1994). Both ρ^+ cells and ρ^0 cells can be further stimulated by the ubiquinone analogues (Larm *et al.*, 1995; this report). In isolated plasma membrane, however, these compounds showed no effect on the NADH:ferricyanide reductase activity but had a dramatic effect on the NADH-oxidase activity. The lack of stimulation of the NADH:ferricyanide reductase activity in isolated plasma membrane may be the result of the loss of a component of the PMOR system during isolation (perhaps ion fluxes, cytosolic or loosely associated protein, or some labile cofactors). Addition of ubiquinone and iron ions did not restore any stimulatory effect of the analogues. It is possible that such addition was not sufficient for the stimulation of the NADH:ferricyanide reductase activity to be observed. A dramatic increase of the NADH-oxidase activity was observed in the presence of the ubiquinone analogues. It was originally proposed that ubiquinone would act as an electron acceptor substrate for the NADH:ferricyanide reductase activity (Martinus *et al.*, 1993). Our extensive data presented here suggest that the NADH-oxidase is the primary target for ubiquinone and its analogues. The NADH-oxidase may, therefore, represent another fundamental aspect of ρ^0 cell survival.

Our use of inhibitors has proven useful in demonstrating that at least two activities can be distinguished. Capsaicin and analogues clearly inhibit the NADH-oxidase activity, but are without effect on the NADH:ferricyanide reductase. Conversely, contrary to previous reports using mouse liver plasma membrane fractions (Morré and Brightman, 1991), pCMBS only affects the NADH:ferricyanide reductase. pCMBS reacts with membrane-associated SH groups and penetrates the plasma membrane very slowly. Further, DTNB has no effect on the reductase activity. Since pCMBS has the ability to react with partially masked SH groups (van Iwaarden *et al.*, 1992), the reactive SH group of the reductase may be partially embedded and therefore less accessible to DTNB. These result suggest that a SH group may be present at the active site of the enzyme.

The work presented here emphasizes the role of ubiquinone in the activity of the PMOR system. Compounds that are closely related to ubiquinone have dramatic effects, either stimulatory (up to 10-fold) or inhibitory (up to 100% inhibition for resiniferatoxin) on the NADH-oxidase activity. While a primary target for these compounds was always assumed to be the mitochondrion, the results presented here show that the PMOR may actually be the primary target. Treatment of pathologies related to mitochondrial impairment (see Nagley *et al.*, 1993; Luft, 1994) has been only anecdotically successful. Ubiquinone, at the level of the plasma membrane, may prove to provide answers to a variety of degenerative pathologies, and could be an important component in treating these conditions.

ACKNOWLEDGMENTS

We are grateful to Prof. F. L. Crane and Dr. M. Degli Esposti for valuable discussions.

REFERENCES

Afanas'ev, I. B., Korkina, L. G., Suslova, T. B., and Soodaeva, S. K. (1990). *Arch. Biochem. Biophys.* **281**, 245–250.

Aledort, L. M., Troup, S. B., and Weed, R. I. (1968). *Blood* **31**, 471–479.

Beaufay, H., Amar-Costesec, A., Feytmans, E., Thinès-Sempoux, D., Wibo, M., Robbi, M., and Berthet, J. (1974). *J. Cell Biol.* **61**, 188–200.

Bérczi, A., Sizensky, J. A., Crane, F. L., and Faulk, W. P. (1991). *Biochim. Biophys. Acta* **1073**, 562–570.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Brandt, U., and Trumpower, B. (1994). *Crit. Rev. Biochem. Mol. Biol.* **29**, 165–197.
Braun, B. S., Benbow, U., Lloyd-Williams, P., Bruce, J. M., and Dutton, P. L. (1986). *Methods Enzymol.* **125**, 119–129.
Brightman, A. O., Wang, J., Miu, R. K.-m., Sun, I. L., Barr, R., Crane, F. L., and Morré, D. J. (1992). *Biochim. Biophys. Acta* **1105**, 109–117.
Clark, M. G., Partick, E. J., Patten, G. S., Crane, F. L., Löw, H., and Grebing, C. (1981). *Biochem. J.* **200**, 565–572.
Crane, F. L., Roberts, H., Linnane, A. W., and Löw, H. (1982). *J. Bioenerg. Biomembr.* **14**, 191–205.
Crane, F. L., Sun, I. L., Clark, M. G., Grebing, C., and Löw, H. (1985). *Biochim. Biophys. Acta* **811**, 233–264.
Crane, F. L., Sun, I. L., Barr, R., and Löw, H. (1991). *J. Bioenerg. Biomembr.* **23**, 773–803.
Crane, F. L., Sun, I. L., and Sun, E. E. (1993). *Clin. Invest.* **71**, S55–S59.
Desjardins, P., Frost, E., and Morais, R. (1985). *Mol. Cell. Biol.* **5**, 1163–1169.
Ellem, K. A. O., and Kay, G. F. (1983). *Biochem. Biophys. Res. Commun.* **112**, 183–190.
Goldenberg, H., Crane, F. L., and Morré, D. J. (1979). *J. Biol. Chem.* **254**, 2491–2498.
Hochstein, P. (1983). *Fundam. Appl. Toxicol.* **3**, 215–217.
Inman, R. S., and Wessling-Resnick, M. (1993). *J. Biol. Chem.* **268**, 8521–8528.
King, M. P., and Attardi, G. (1989). *Science* **246**, 500–503.
Larm, J. A., Vaillant, F., Linnane, A. W., and Lawen, A. (1994). *J. Biol. Chem.* **269**, 30097–30100.
Larm, J. A., Wolvetang, E. J., Vaillant, F., Martinus, R. D., Lawen, A., and Linnane, A. W. (1995). *Protoplasma* **184**, 173–180.
Lawen, A., Martinus, R. D., McMullen, G. L., Nagley, P., Vaillant, F., Wolvetang, E. J., and Linnane, A. W. (1994). *Mol. Aspects Med.* **15**, s13–s27.
Lenaz, G., Pasquali, P., Bertoli, E., and Parenti-Castelli, G. (1975). *Arch. Biochem. Biophys.* **169**, 217–226.
Löw, H., Grebing, C., Lindgren, A., Tally, M., Sun, I. L., and Crane, F. L. (1987). *J. Bioenerg. Biomembr.* **19**, 535–549.
Luft, R. (1994). *Proc. Natl. Acad. Sci. USA* **91**, 8731–8738.
Mahler, H. R. (1955). *Methods Enzymol.* **2**, 688–693.
Martinus, R. D., Linnane, A. W., and Nagley, P. (1993). *Biochem. Mol. Biol. Int.* **31**, 997–1005.
Morré, D. J., and Brightman, A. O. (1991). *J. Bioenerg. Biomembr.* **23**, 469–489.
Morré, D. J., and Morré, D. M. (1989). *BioTechniques* **7**, 946–958.
Nagley, P., Zhang, C., Martinus, R. D., Vaillant, F., and Linnane, A. W. (1993). In *Mitochondrial DNA in Human Pathology* (DiMauro, S., and Wallace, D. C., eds.), Raven Press, New York, pp. 137–157.
Naruta, Y. (1980). *J. Am. Chem. Soc.* **102**, 3774–3783.
Navas, P., Estévez, A., Burón, M. I., Villalba, J. M., and Crane, F. L. (1988). *Biochem. Biophys. Res. Commun.* **154**, 1029–1033.
Navas, P., Nowack, D. D., and Morré, D. J. (1989). *Cancer Res.* **49**, 2147–2156.
Nishinaka, Y., Aramaki, Y., Yoshida, H., Masuya, H., Sugawara, T., and Ichimori, Y. (1993). *Biochem. Biophys. Res. Commun.* **193**, 554–559.
Nyormoi, O., Klein, G., Adams, A., and Dombos, L. (1973). *Int. J. Cancer* **12**, 396–408.
Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967). *J. Cell Biol.* **32**, 415–438.
Sun, I. L., Toole-Simms, W., Crane, F. L., Golub, E. S., Díaz de Pagán, T., Morré, D. J., and Löw, H. (1987). *Biochem. Biophys. Res. Commun.* **146**, 976–982.
Sun, I. L., Crane, F. L., Grebing, C., and Löw, H. (1984a). *J. Bioenerg. Biomembr.* **16**, 583–595.
Sun, I. L., Crane, F. L., Löw, H., and Grebing, C. (1984b). *J. Bioenerg. Biomembr.* **16**, 209–221.
Sun, I. L., Sun, E. E., and Crane, F. L. (1992a). *Biochem. Biophys. Res. Commun.* **189**, 8–13.
Sun, I. L., Sun, E. E., Crane, F. L., Morré, D. J., Lindgren, A., and Löw, H. (1992b). *Proc. Natl. Acad. Sci. USA* **89**, 11126–11130.
Sun, I. L., Sun, E. E., and Crane, F. L. (1995). *Protoplasma* **184**, 214–219.
Vaillant, F., and Nagley, P. (1995). *Hum. Mol. Genet.* **4**, 903–914.
Vaillant, F., Loveland, B. E., Nagley, P., and Linnane, A. W. (1991). *Biochem. Int.* **23**, 571–580.
van Iwaarden, P. R., Driessen, A. J. M., and Konings, W. N. (1992). *Biochim. Biophys. Acta* **1113**, 161–170.
VanSteveninck, J., Weed, R. I., and Rothstein, A. (1965). *J. Gen. Physiol.* **48**, 617–632.
Villalba, J. M., Navarro, F., Córdoba, F., Serrano, A., Arroyo, A., Crane, F. L., and Navas, P. (1995). *Proc. Natl. Acad. Sci. USA* **92**, 4887–4891.
Wan, Y.-P., Williams, R. H., Folkers, K., Leung, K. H., and Racker, E. (1975). *Biochem. Biophys. Res. Commun.* **63**, 11–15.
Wanders, R. J. A., Kos, M., Roest, B., Meijer, A. J., Schrakamp, G., Heymans, H. S. A., Tegelaers, W. H. H., van den Bosch, H., Schutgens, R. B. H., and Tager, J. M. (1984). *Biochem. Biophys. Res. Commun.* **123**, 1054–1061.
Wolvetang, E. J., Johnson, K. L., Krauer, K., Ralph, S. J., and Linnane, A. W. (1994). *FEBS Lett.* **339**, 40–44.
Wolvetang, E. J., Larm, J. A., Mousoulas, P. and Lawen, A. (1996). *Cell Growth Differ.* **7**, in press.
Wróblewski, F., and LaDue, J. S. (1955). *Proc. Soc. Exp. Biol. Med.* **90**, 210–213.