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Regulation of the Mitochondrial Permeability Transition Pore by Ubiquinone Analogs. A Progress Report

LUDIVINE WALTER[a], HIDETO MIYOSHI[b], XAVIER LEVERVE[a], PAOLO BERNARDI[c,*] and ERIC FONTAINE[a]

[a]*Laboratoire de Bioénergétique Fondamentale et Appliquée, Université Joseph Fourier, F-38041 Grenoble-cedex 09, France;* [b]*Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan;* [c]*Consiglio Nazionale delle Ricerche Unit for the Study of Biomembranes and Department of Biomedical Sciences, University of Padova Medical School, Viale G. Colombo 3, I-35121 Padova, Italy*

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The permeability transition pore (PTP) is a mitochondrial inner membrane Ca^{2+}-sensitive channel that plays a key role in different models of cell death. In a series of recent studies we have shown that the PTP is modulated by quinones, and we have identified three functional classes: (i) PTP inhibitors; (ii) PTP inducers; and (iii) PTP-inactive quinones that compete with both inhibitors and inducers. Here, we review our current understanding of pore regulation by quinones, and present the results obtained with a new series of structural variants. Based on the effects of the compounds studied so far, we confirm that minor structural changes profoundly modify the effects of quinones on the PTP. On the other hand, quinones with very different structural features may have qualitatively similar effects on the PTP. Taken together, these results support our original proposal that quinones affect the PTP through a common binding site whose occupancy modulates its open–closed transitions, possibly through secondary changes of the Ca^{2+}-binding affinity.

Keywords: Mitochondria; Quinones; Permeability transition; Cell death

Abbreviations: PT, Permeability transition; PTP, Permeability transition pore; CsA, Cyclosporin A; CRC, Ca^{2+} Retention capacity; Ub_0, Ubiquinone 0; Ub_5, Ubiquinone 5; Ub_{10}, Ubiquinone 10; 2,3-EtO-Ub_{10}, 2,3-diethoxy-Ubiquinone 10; 3-BuO-Ub_{10}, 3-*n*-buthoxy-Ubiquinone 10; 2,3-Me-Ub_{10}, 2,3-dimethyl-Ubiquinone 10; DUb, Decylubiquinone; 5-H-DUb, 5-H-Decylubiquinone; 5-Et-DUb, 5-ethyl-Decylubiquinone; 2-EtO-DUb, 2-ethoxy-Decylubiquinone; 3-EtO-DUb, 5-ethoxy-Decylubiquinone; 2,3-Me-5-H-DUb, 2,3-dimethyl-5H-Decylubiquinone; 2,3-EtO-PUb, 2,3-diethoxy-Pentylubiquinone; (OH)DUb, hydroxy-Decylubiquinone; PUb, Pentylubiquinone; 2,3,5-Me-6(OH)iP-Bz, 2,3,5-trimethyl-6-(3-hydroxy-isopentyl)-Benzoquinone; 5-H-(Et)BUb, 5-H-(1-ethyl)-Butylubiquinone; 2,5-OH-3-H-UUb, 2,5-dihydroxy-3-H-Undecylubiquinone

INTRODUCTION

Regulation of ion fluxes across the inner mitochondrial membrane is essential both for metabolic regulation and for energy conservation. The inner membrane possesses an intrinsically low permeability to ions and solutes, which allows energy conservation in the form of a proton electrochemical potential difference.[1] Yet, mitochondria *in vitro* can easily undergo a permeability increase to solutes with molecular masses of about 1500 Da or lower, which is followed by deenergization, disruption of ionic homeostasis and matrix swelling. This event, called the "permeability transition" (PT), is due to the opening of an inner membrane pore, the PTP (see Ref. [2] for review). Recent years have witnessed an exponential increase of the interest in the PT as a potential mediator of cell death both in the context of necrosis and of apoptosis (see Ref. [3] for review). The PT could play a role by causing ATP depletion and dysregulation of Ca^{2+} homeostasis, and/or by contributing to the release of cytochrome *c*[4] and smac/diablo[5,6] through matrix swelling and possibly outer membrane rupture.[7]

The discovery of the effects of Cyclosporin A (CsA), which in isolated mitochondria inhibits the PT with a K_i of about 10 nM (e.g. Ref. [8]), prompted extensive investigations of the putative role of the PTP in cell, organ as well as in animal models of disease. These studies tried to assess

*Corresponding author. E-mail: bernardi@civ.bio.unipd.it

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whether the PT represents a potential factor for mitochondrial dysfunction, and the results are particularly convincing in the context of brain injury and heart ischemia. Increasing evidence indicates that PTP opening may be a key event in the tissue damage that follows forebrain ischemia,[9,10] hypoglycemic coma,[11,12] traumatic brain injury[13,14] as well as global ischemia-reperfusion of the heart.[15] CsA displays a dramatic protective effect in all these models[10–12,15] indicating that the PT represents a viable target for pharmacological intervention.[2] CsA has multiple effects on cells, particularly in relation to inhibition of the cytosolic phosphatase calcineurin, which is involved in nuclear translocation of NF-AT and is responsible for the immunosuppressive effects.[16] Derivatives devoid of effects on calcineurin but still active on the PT have been described, such as MeVal-4-cyclosporin,[8] which protects the heart from ischemia-reperfusion damage,[15] and hippocampal neurons from damage caused by glucose/oxygen deprivation.[17] Even this derivative, however, inhibits all cellular cyclophilins. Furthermore, CsA does not readily cross the blood–brain barrier, and this would limit its usefulness in a clinical setting. Taken together, these observations point at PTP opening as a pathogenetic factor in several diseases, and at the importance of developing novel PTP-active drugs.

We have long been involved in the study and characterization of PTP inducers and inhibitors, with the long-term goals of defining the PTP regulatory features and molecular nature, and of developing better drugs for its modulation *in vivo*. In a series of recent studies we have shown that the PTP is modulated by electron flux through respiratory chain Complex I,[18] which led to the discovery that the PT is inhibited by Ub_0 and DUb.[19] Inhibition by these quinones could be specifically relieved by Ub_5, which is inactive *per se*, suggesting that quinones could be competing for a common binding site.[19] Similar findings have been recently described in an elegant set of experiments on the mitochondrial megachannel, the electrophysiological counterpart of the PTP.[20] To identify the structural features of ubiquinones required for regulation of the PTP, we have carried out a detailed analysis with several structural variants.[19,21] These studies are reviewed below, where we also present the effects of a new series of compounds and a comparison of their relative potency on PTP modulation.

THREE CLASSES OF PTP-MODULATING QUINONES

Of all the quinones we tested so far, 18 compounds could be shown to affect the PTP. Their structures are summarized in Fig. 1, where the quinones are grouped according to the three functional classes that we have identified: Group I (inhibitors); Group II (inducers); and Group III (apparently inactive quinones that compete with both inhibitors and inducers).[21]

We have studied the relative efficacy of Group I and Group II quinones at PTP inhibition and induction, respectively, by determining their effects on the mitochondrial Ca^{2+} retention capacity (CRC), a measure of the minimal Ca^{2+} load required to induce PTP opening. For comparative purposes CsA, the standard high-affinity PTP inhibitor, was also included in these assays. Figure 2 shows that the 11 Group I quinones inhibit the PTP with different potency. Ub_0 was the more potent, and more efficient than CsA while the other compounds (2,3-EtO-PUb; 5-H-(Et)BUb; DUb; 5-Et-DUb; 3-EtO-DUb; 2,3-Me-5-H-DUb; Ub_{10}; 2,3-EtO-Ub_{10}; 3-BuO-Ub_{10}; 2,3-Me-Ub_{10}) displayed a variable level of efficacy. It should be noted that for each compound we used the concentration causing a maximal effect, and that the efficacy should be intended in terms of the biological effect rather than on a molar basis.

It should be stressed that inhibition by Group I quinones is not limited to the PT induced by Ca^{2+} plus Pi, but it is also observed with other inducers such as atractylate, oxidative stress and mitochondrial depolarization indicating that quinones are general inhibitors of the PT.[19] The concentration-dependence of the effects of Group I quinones on the CRC is quite unusual. Indeed, these compounds increase the CRC sigmoidally, reach an optimum concentration which differs for the different quinones, and at higher concentrations display a decreased efficiency, and may even become inducers rather than inhibitors (see Fig. 3 and Refs. [18,19,21]). Although not well understood at present, this paradoxical behavior is of great practical interest because the effective quinone "concentrations" that are reached *in situ*, and hence their cellular effects, will dictate the prevailing effects on the PTP. This issue will be discussed more in detail below.

The Group II quinones are compounds that favor pore opening in the sense that a lower Ca^{2+} load is required to trigger a PT in their presence, as shown in Fig. 2 for 2,5-OH-3-H-UUb and (OH)Dub. The concentration-dependence of the effects of Group II quinones is simpler than that of Group I quinones, in the sense that they cause a monotonic decrease of the CRC.[21]

Group III quinones are able to compete with Group I and/or Group II quinones at concentrations that do not, *per se*, affect the CRC. Yet, and as previously emphasized,[19] some Group I quinones like Ub_{10} and 2,3-EtO-Ub_{10} behave as PT inducers at very high concentrations.

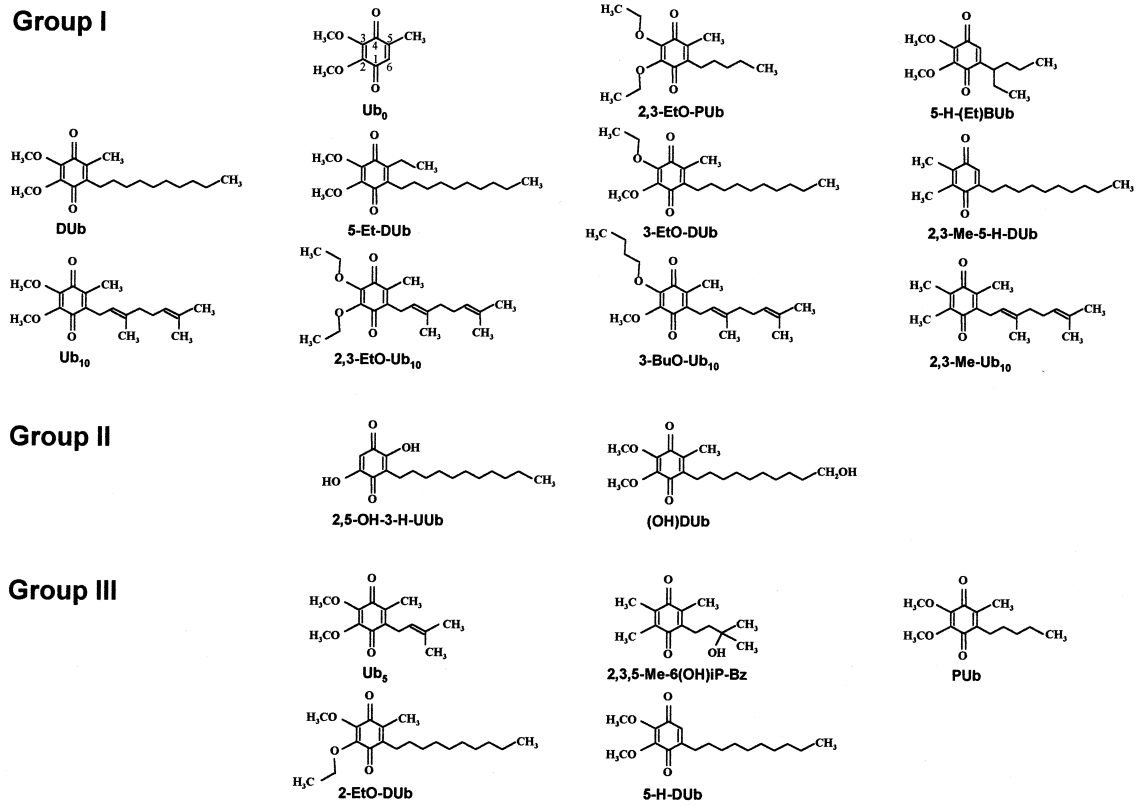

FIGURE 1 Chemical structure of PTP-active ubiquinone analogs. Groups I, II and III denote PTP-inhibiting, PTP-inducing and PTP-binding analogs that have no effects *per se* but counteract the effects of both Group I and II quinones.

COMPETITION BETWEEN QUINONES

The 18 PTP-active quinones have been discovered after a careful screening of more than 70 ubiquinone analogs. The fact that specific chemical features were required for PTP regulation, and the "competition" for PTP modulation, led us to propose that regulatory quinones may bind to the pore, making it either less (Group I quinones) or more (Group II quinones) sensitive to Ca^{2+}. Since binding to the pore does not predict the way the PT is regulated, we also hypothesized that PTP-inactive quinones may bind to the pore without affecting its open–closed transitions.[22] In order to test this hypothesis, we have studied the effects of combinations of quinones on either the CRC, or on the swelling that follows a PT *in vitro*.

Figure 4 illustrates typical protocols used to study quinone interactions with the PTP, and reports the effects of the novel derivative 5-H-DUb. Panel A shows that the addition of 150 μM Ca^{2+} caused PTP opening, monitored here as the decrease of absorbance at 540 nm (trace a). PTP opening was prevented by 50 μM Ub_{10} (traces b and c), while the addition of the Group III quinone 5-H-DUb readily caused pore opening (trace c), thus removing the inhibitory effects of Ub_{10}. The experiments of panel B show that in the presence of 100 μM Ub_{10} the addition of 50 μM Ca^{2+} readily caused PTP opening (trace b), while the same Ca^{2+} load was ineffective in the absence of Ub_{10} (trace a), indicating that at this concentration Ub_{10} behaves like an inducer rather than as inhibitor (see also Fig. 2). On the other hand, the Group III quinone 5-H-DUb again counteracted the effects of Ub_{10} and prevented PTP opening by 50 μM Ca^{2+} (trace c).

Table I summarizes the results we obtained in competition experiments of this type. Due to limited availability of some quinones not all possible combinations could be tested, yet the results clearly document that not all Group III quinones at the concentration used here compete will all PTP-active quinones. For example 2,3-EtO-Pub and DUb compete with all Group III quinones, whereas no Group III quinone competes with 2,5-OH-3-H-UUb. Assuming that the Group I, II and III quinones share the same binding site on the PTP (see below for discussion), these results suggest that quinones compete with each other according to their relative local concentration and to their binding affinities. Within Group III, it is conceivable that quinones with the largest spectrum of "competitive" activity may have a higher binding affinity than those with a more limited spectrum. Finally, although not all possible combinations could be tested, it should be noted that the Group II, PTP-inducing quinones can compete with all the tested Group I quinones.[19,21] We would

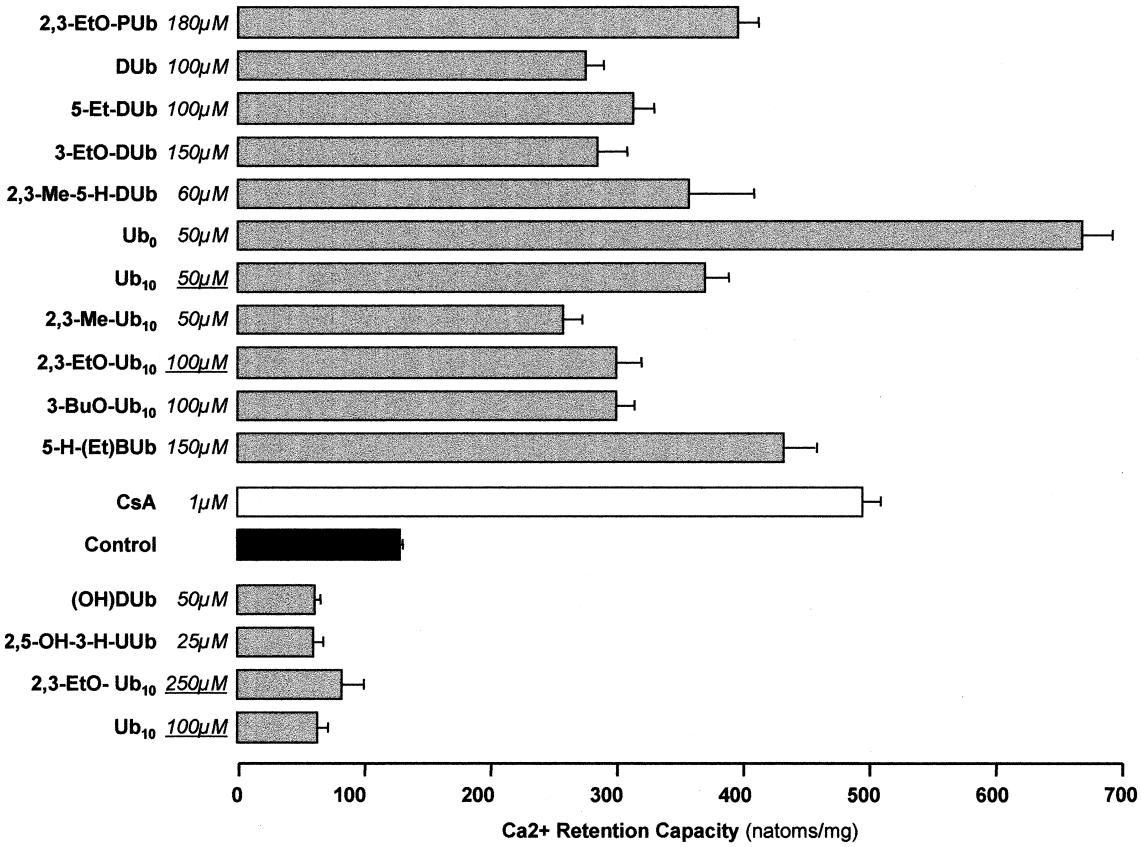

FIGURE 2 Effect of quinones on the Ca^{2+} retention capacity of rat liver mitochondria. The incubation medium contained 250 mM sucrose, 1 mM Pi-Tris, 10 mM Tris-MOPS, 5 mM glutamate-Tris, 2.5 mM malate-Tris, 1 μM Calcium Green-5N. The final volume was 2 ml, pH 7.4, 25°C. Experiments were started by the addition of 2 mg of mitochondria, followed by the indicated concentrations of PTP-active ubiquinone analogs (gray bars) or of CsA (open bar). Black bar, no further additions. Trains of 25 μM Ca^{2+} pulses were added at 1 min intervals, and the Ca^{2+} retention capacity denotes the amount of Ca^{2+} necessary to induce a PT. Results are mean ± S.E. ($n = 6$). For further experimental details and protocols see Refs. [19,21].

like to stress that competition between the Group I quinones Ub_0 and DUb and the Group III quinone Ub_5 has also been observed at the single channel level.[20]

It should be noted that in these experiments "competition" simply refers to the ability of a given quinone to revert the effects of another quinone, and that the interesting issue of whether the interaction of quinones of the three groups is competitive or non-competitive in nature could not be conclusively addressed.

In principle, all the PTP-binding quinones sharing a unique binding site could interact with one another, and a competitive interaction would shift the EC_{50}. In practice, however, the apparent EC_{50} values obtained in this type of experiments are not entirely reliable. Indeed, in the presence of optimal concentrations of Group I quinones addition of Group III quinones does decrease the CRC, but the subsequent addition of Group I quinones does not restore the initial CRC because the latter quinones become less active at these higher concentrations.

HOW MANY QUINONE BINDING SITES?

A key issue remains the mechanism through which quinones modulate the PTP. In principle, the existence of inducing and inhibiting quinones could be explained by the existence of two quinone-binding sites, one responsible for inhibition and one for activation. Assuming that Group I quinones may also bind to the latter site with lower affinity, the biphasic response observed could be easily explained because high concentrations of Group I quinones could indeed open the pore through the second site. This would be consistent with the response to Ub_{10} and 2,3EtO-Ub_{10} but not to other Group I quinones, which lose their activity but do not become activators at high concentrations.

Although the existence of different sites cannot be conclusively ruled out at present, we favor a model where the competition between Group I, II and III quinones is mediated by binding to a common site, whose occupancy would in turn modulate the PTP open–closed transitions through secondary changes of the PTP Ca^{2+}-binding affinity. The scheme of Fig. 5

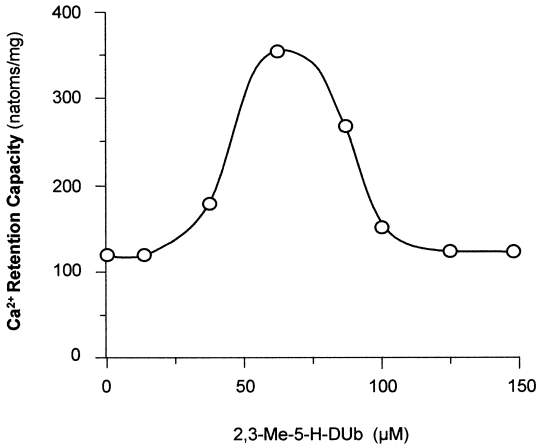

FIGURE 3 Ca^{2+} retention capacity of rat liver mitochondria at increasing concentration of the Group I quinone 2,3-Me-5-H-DUb. Experimental conditions were as in Fig. 2, except that the indicated concentrations of 2,3-Me-5-H-DUb were present.

depicts our current working model to explain how quinones may regulate the Ca^{2+}-dependent PTP open–closed transitions. We hypothesize that the PTP can exist in a liganded state with either inducing, inactive or inhibiting quinones, which in turn would confer different conformations to the pore that may correspond to states with different accessibility to Ca^{2+} ions. In this scenario, addition of a small Ca^{2+} load can cause the transition from the closed state to the open state only if the PTP is liganded with inducing, Group II quinones. A larger Ca^{2+} load would be required to access the Ca^{2+} binding site(s) in the conformation liganded with inactive, Group III quinones and a still larger Ca^{2+} load in the state liganded with inhibitory, Group I quinones. When several quinones are present, they would compete with each other according to their relative local concentration and to their binding affinities, resulting in turn in a subtle modulation of the accessibility to Ca^{2+} and therefore of the PTP open–closed transitions.

This model can also account for why Group I quinones become less active as their concentration is increased. Due to their high hydrophobicity, quinones tend to accumulate in mitochondrial membranes, and only a small proportion is soluble in water where quinones organize in nonmonomeric states when their critical micellar concentration is exceeded. Since the properties of quinones are likely to change when they are in nonmonomeric states, we suggest (i) that monomeric quinones are most effective at PTP inhibition, and (ii) that nonmonomeric quinones may bind to the pore and compete with quinones in the monomeric state. PTP-inactive complexes would displace the PTP-inhibitory monomers, thus abolishing their protective effects, whereas PTP-inducing complexes would still trigger PTP opening. Although entirely hypothetical, this explanation has the merit to account for the paradoxical decrease of potency of increasing concentrations of inhibitory quinones on the mitochondrial CRC.

WHAT IS THE STRUCTURE–FUNCTION CORRELATION?

Since many quinone analogs do not regulate the PTP,[19,21] the 1,4-benzoquinone ring *per se* is not sufficient for pore regulation, and specific substituents on carbons 2,3,5 and 6 may be essential for PTP binding and regulation. On one hand, it is striking that even minor changes in chemical structure

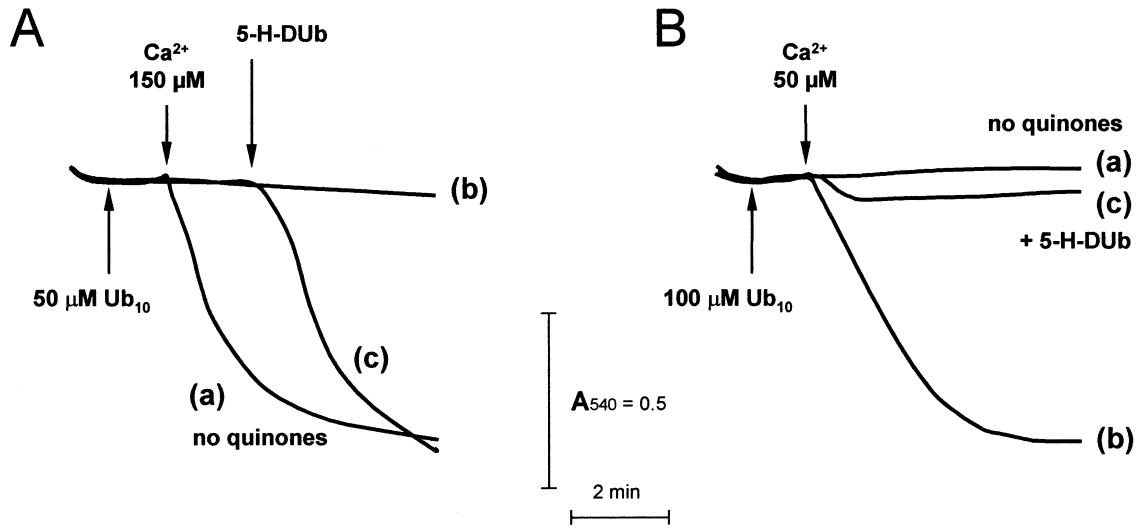

FIGURE 4 Effect of the Group III quinone, 5-H-DUb on PTP regulation by Ub_{10}. Experimental conditions were as in Fig. 2, but PTP opening was detected based on the absorbance decrease of the mitochondrial suspension at 540 nm. The experiments were started by the addition of 2 mg of mitochondria (not shown). Panel A: Where indicated 50 μM Ub_{10} was added (traces b and c only), followed by Ca^{2+} (all traces) and by 150 μM 5-H-DUb (trace c only). Panel B: Right after the addition of mitochondria 150 μM 5-H-DUb was added (not shown, trace c only); where indicated 100 μM Ub_{10} was added (traces b and c only) followed by Ca^{2+} (all traces).

TABLE I Competition between quinone analogs. Experimental conditions were as in Fig. 4. The experiments were started by the addition of 2 mg of mitochondria in the presence of the indicated concentrations of quinones. When inhibitory Group I quinones were tested, mitochondria where loaded with 150 μM Ca^{2+} and exposed to 150 μM of the Group III quinones. When inducing Group II quinones were tested, mitochondria where co-incubated in the presence of 150 μM Group III quinones and exposed to 50 μM Ca^{2+}. Competition between Group I and III quinones was revealed by PTP opening after the addition of Group III quinones, whereas competition between Group II and III quinones was revealed by lack of PTP opening after Ca^{2+} addition. +, competition, 0, no competition, n.d., not determined

Quinone	[C] (μM)	Ub_5	2-EtO-DUb	5-H-Dub	2,3,5-Me-6-(OH)iP-Bz	PUb
2,3-EtO-PUb	180	+	+	+	+	+
DUb	100	+	+	+	+	+
5-Et-DUb	100	+	+	n.d.	+	n.d.
3-EtO-DUb	150	n.d.	+	n.d.	n.d.	n.d.
2,3-Me-5-H-DUb	60	+	n.d.	n.d.	0	n.d.
Ub_0	50	+	+	+	0	+
Ub_{10}	50	+	+	+	+	0
2,3-Me-Ub_{10}	50	+	n.d.	n.d.	0	n.d.
2,3-EtO-Ub_{10}	100	n.d.	n.d.	n.d.	n.d.	+
3-BuO-Ub_{10}	100	+	0	n.d.	0	n.d.
5-H-(Et)BUb	150	0	+	0	0	n.d.
(OH)DUb	50	+	+	0	0	0
2,5-OH-3-H-UUb	25	0	0	0	0	0
2,3-EtO-Ub_{10}	250	0	0	n.d.	0	n.d.
Ub_{10}	100	0	+	+	0	0

profoundly affect the quinone interactions with the pore. For instance, although 3-EtO-DUb and 2-EtO-DUb are isomeric PTP-binding quinones, the former is an inhibitor while the latter is inactive; and replacement of a decyl group with a 10′-hydroxy-decyl group transforms an inhibitor (DUb) into an inducer [(OH)DUb]. On the other hand, quinones possessing very different chemical structures have similar effect on PTP regulation (see Fig. 1) suggesting that the quinone spatial conformation may be more important than the nature of the substituents *per se*. Although we have not, as of today, identified the key features that affect the correlation between quinone structure and effects on the PTP it is clear that neither the redox potential of quinones nor the hydrophobicity of the side chain are crucial factor for the PTP regulation, as we suggested in earlier studies.[18,19,21,22]

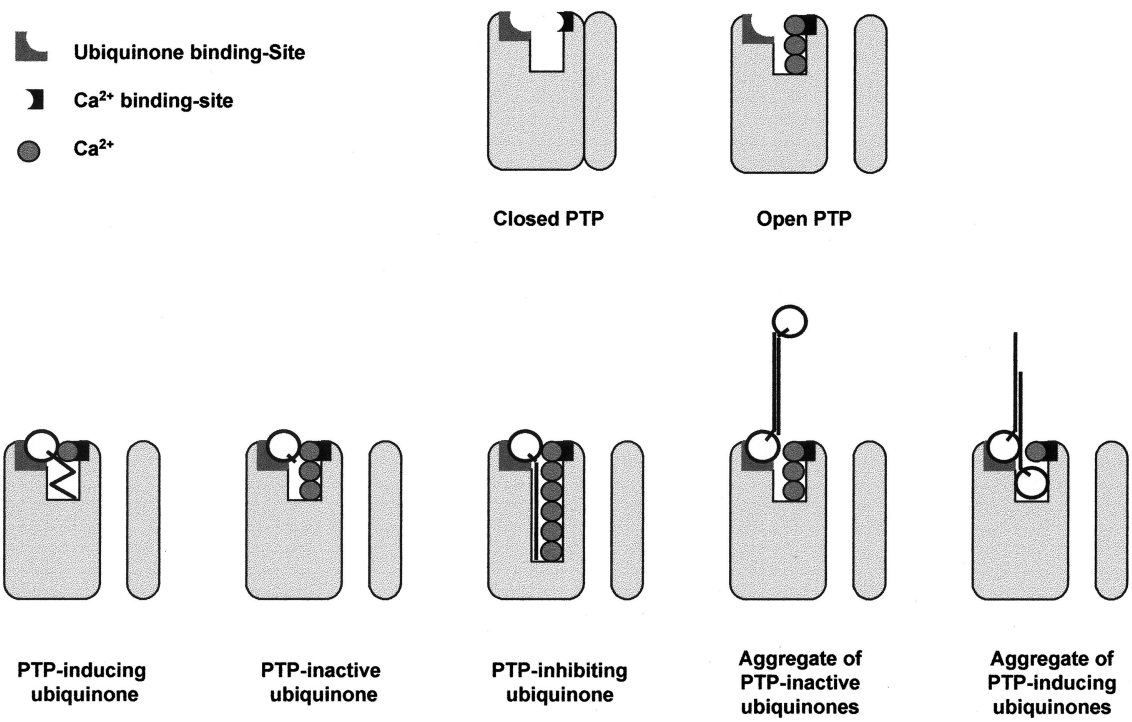

FIGURE 5 Model for PTP regulation by ubiquinones analogs. The open space between the rectangles denotes the open state of the PTP. For explanation, see text.

OTHER EFFECTS OF UBIQUINONE ANALOGS ON MITOCHONDRIA

Besides their effects on the PTP, ubiquinone analogs interfere with electron transfer through the respiratory chain. As artificial electron acceptors, they oxidize mitochondrial NADH (e.g. Ref. [18]) and decrease reactive oxygen species formation (e.g. Ref. [21]). Some quinones inhibit mitochondrial respiration.[23] However, none of these effects univocally correlates with their actions on the pore, suggesting that the latter rather depend on direct interactions that remain to be fully understood.

SUMMARY AND PERSPECTIVES

We have reviewed the features of PTP regulation by three classes of ubiquinone analogs in isolated mitochondria. The discovery of this novel class of PTP-active compounds has several interesting implications because it provides: (i) an important clue on the nature of the PTP, which might be closely related to the respiratory chain Complexes, as discussed in a recent review;[22] (ii) an intriguing target for the pharmacological effects of quinones *in vivo*, which have been shown to be protective in several forms of disease;[24–29] and (iii) an original framework for the synthesis of novel drugs, which will in turn allow a better refinement of the structure function-correlation. These studies are currently under way in our laboratories, and hold great promise for a therapeutical strategy in diseases where pore opening appears to play a major role.[15]

Acknowledgements

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