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INTERACTION OF BUTYLATED HYDROXYANISOLE WITH MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

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Abstract—The antioxidant, butylated hydroxyanisole (BHA), has a number of effects on mitochondrial oxidative phosphorylation. In this study we apply the novel approach developed by Brand (Brand MD, *Biochim Biophys Acta* **1018**: 128–133, 1990) to investigate the site of action of BHA on oxidative phosphorylation in rat liver mitochondria. Using this approach we show that BHA increases the proton leak through the mitochondrial inner membrane and that it also inhibits the Δp (proton motive force across the mitochondrial inner membrane) generating system, but has no effect on the phosphorylation system. This demonstrates that compounds having pleiotypic effects on mitochondrial oxidative phosphorylation *in vitro* can be analysed and their many effects distinguished. This approach is of general use in analysing many other compounds of pharmacological interest which interact with mitochondria. The implications of these results for the mechanism of interaction of BHA with mitochondrial oxidative phosphorylation are discussed.

Butylated hydroxyanisole (BHA§) is widely used as an antioxidant food additive (E320) [1]. At high concentrations it is toxic to human cell lines [2] and to isolated hepatocytes [3]. This toxicity is believed to be due to the inhibition of respiration by BHA which has been demonstrated in cell lines [4] and in the protozoon *Trypanosoma cruzi* [5]. BHA has a number of effects on isolated mitochondria: it stimulates state 4 respiration, inhibits state 3 and uncoupled respiration and decreases the mitochondrial membrane potential [3, 6]. These results suggest that BHA may have multiple effects on mitochondria. In this study we determined the components of the mitochondrial oxidative phosphorylation system which are affected by BHA. To do this we adopted the rigorous approach recently developed by Brand [7]. This approach has been used to isolate the sites of action of glucagon and thyroid hormone on mitochondria [8–10]. It is ideally suited to analyse compounds having pleiotypic effects on oxidative phosphorylation and may be of general utility in determining how compounds of pharmacological interest affect mitochondria. This is because effects on the respiratory chain and substrate transporters, the proton conductivity of the mitochondrial inner membrane and on the mitochondrial phosphorylation system can be separated from each other. BHA, which has multiple effects on mitochondria, is a good compound with which to test this approach and here we adapt this technique to determine the sites of action of BHA on isolated mitochondria *in vitro*.

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§ Abbreviations: Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; BHA, butylated hydroxyanisole (2-*t*-butyl-4-methoxyphenol); FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; $\Delta\psi$, electrical potential across the mitochondrial inner membrane; Δp, proton motive force across the mitochondrial inner membrane; TPMP, methyltriphenylphosphonium.

MATERIALS AND METHODS

Isolation of mitochondria. Liver mitochondria from male Wistar rats of 200–250 g were prepared by homogenization followed by differential centrifugation [11] in ice-cold medium containing 250 mM sucrose, 5 mM Tris and 1 mM EGTA adjusted to pH 7.4 (HCl) at 25°. The mitochondrial protein content was determined by the biuret method using bovine serum albumin as a standard [12].

Measurement of respiration rate and $\Delta\psi$. The respiration rate was measured using an oxygen electrode (Rank Brothers, Bottisham, Cambridgeshire, U.K.). Respiration rate was calculated assuming an oxygen concentration of 475 nmol O/mL in the experimental medium at 25° [13]. The mitochondrial $\Delta\psi$ was determined from the distribution of the lipophilic cation methyltriphenylphosphonium (TPMP) across the mitochondrial inner membrane [14]. The membrane potential was then calculated from the Nernst equation assuming that 57% of the matrix TPMP was bound and that BHA did not affect TPMP binding [9]. The distribution of TPMP between the mitochondrial matrix and the external medium was measured using a TPMP-sensitive electrode [15]. The TPMP electrode was inserted through the perspex lid of the oxygen electrode chamber enabling the simultaneous measurement of membrane potential and respiration rate which is essential for these experiments. All experiments were carried out in the presence of the ionophore nigericin and 120 mM

KCl. This clamped the pH gradient across the mitochondrial inner membrane close to zero [16, 17], therefore $\Delta\psi$ was the sole component of Δp.

Measurement of mitochondrial volume. The mitochondrial volume was determined using [^{14}C]-sucrose as an extramitochondrial marker and ^3H_2O to determine the total pellet volume [16, 18]. Varying the concentrations of inhibitors or uncouplers over the range used in these experiments had no effect on mitochondrial volume. However, 100 μM BHA decreased the mitochondrial volume slightly compared to control. In control experiments, the mitochondrial volume was 0.70 μL/mg of mitochondrial protein (three different mitochondrial preparations; SEM = 0.05 μL/mg) whereas in the presence of BHA the matrix volume was 0.62 μL/mg of mitochondrial protein (three different mitochondrial incubations; SEM = 0.02 μL/mg). These mitochondrial matrix volumes in the presence of BHA or control were used to calculate $\Delta\psi$.

Measurement of the rate of ATP synthesis. Mitochondrial ATP production was measured as the rate of glucose-6-phosphate synthesis. Respiration rate and $\Delta\psi$ were measured simultaneously allowing the rate of ATP synthesis to be measured as a function of $\Delta\psi$. Hexokinase (10 U) was present and P^1, P^5-di(adenosine-5') pentaphosphate (Ap_5A; 500 μM; Li^+ salt) was added to inhibit adenylate kinase [19]. Phosphorylation was terminated by the addition of oligomycin (1.65 μg/mL). This caused the expected increase in $\Delta\psi$ and decrease in respiration rate. Duplicate 0.25 mL samples were then taken and deproteinized in ice-cold 2.5 M $HClO_4$/50 mM EDTA [20]. The denatured protein was pelleted by brief centrifugation in a bench-top centrifuge and the supernatant was removed and neutralized with ice-cold 5 M KOH/0.3 M 3-[*N*-morpholino]propane-sulphonic acid (Mops) [20]. The $KClO_4$ was pelleted by centrifugation and the supernatant was assayed for glucose-6-phosphate enzymatically [21]. In control experiments the rate of glucose-6-phosphate synthesis was linear with time for at least 6 min after the initiation of respiration and was proportional to the amount of mitochondrial protein up to at least 3 mg of mitochondrial protein/mL. Oligomycin completely inhibited ATP synthesis by energized mitochondria, therefore 500 μM Ap_5A eliminates ATP synthesis by adenylate kinase. Injection of oligomycin into the reaction chamber stops ATP synthesis immediately; the glucose-6-phosphate content on injection of oligomycin was unchanged 6 min later. This procedure can therefore be used to terminate phosphorylation. For all experiments reported here ATP synthesis was stopped with oligomycin 4 min after the initiation of respiration.

Materials. BHA was from Fluka A. G. (Buchs, Switzerland) and was recrystallized once from petroleum ether and once from ethanol prior to use. A stock solution of BHA (30 mM in ethanol) was stored at 4°, shielded from light with aluminium foil. [^{14}C]Sucrose and ^3H_2O were from Amersham Internaional. The scintillant "Ecolite^{TM} (+)" was from ICN Biomedicals Ltd. All other chemicals and enzymes were from Sigma or BDH.

Experimental protocol. Mitochondria (6 mg of mitochondrial protein) were suspended in 3 mL of medium [120 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 10 mM Hepes, 1 mM EGTA, 10 mM glucose, 13.3 μM rotenone and nigericin (0.2 μg/mL); pH 7.2 (KOH) at 25°] in an air-tight, rapidly stirred 3 mL chamber thermostatted at 25°. For some experiments further additions of oligomycin (1.65 μg/mL), ADP (200 μM), hexokinase (10 U) or Ap_5A (500 μM) were made at this stage. Five minutes later 100 μM BHA (or vehicle) was added and additions of TPMP were made to calibrate the TPMP electrode giving a final concentration of 5 μM TPMP. Four minutes after the addition of BHA 5 mM succinate (K^+ salt) was added, this was followed by additions of inhibitors or uncouplers as outlined in the figure legends. At the end of the incubation carbonyl-cyanide - *p* - trifluouromethoxyphenylhydrazone (FCCP; 0.33 μM) was added to allow the TPMP electrode trace to return to its baseline. The respiration rate and $\Delta\psi$ were determined for each new steady state brought about by the addition of uncoupler or inhibitor. Results are presented as mean ± SEM of N experiments and statistical significance was determined using Student's *t*-test.

RESULTS

Effects of BHA on the Δp *producing system*

If non-phosphorylating mitochondria are titrated with an uncoupler to increase the rate of respiration and decrease $\Delta\psi$, a plot of $\Delta\psi$ against respiration rate can be constructed. This plot is a description of the kinetic response of the Δp producers (i.e. the electron transport chain and the substrate transporters) to their product, Δp [8]. Therefore, if a compound inhibits any component of the Δp producing system this curve will be displaced downwards and to the left. That is, at a given value of Δp the respiration rate will decrease. By titrations such as this we can determine whether a compound inhibits the Δp producers. This eliminates any secondary effects of the compound on respiration via alterations in Δp. Figure 1 shows plots of $\Delta\psi$ against respiration rate in the presence of BHA or control. Nigericin and 120 mM KCl eliminated the pH gradient across the mitochondrial inner membrane [16, 17] and therefore $\Delta\psi$ is equal to Δp. In Fig. 1, the presence of BHA displaces the titration of $\Delta\psi$ against respiration rate downwards and to the left. Therefore BHA inhibits one or more of the components of the Δp generating system. Under these conditions the stimulation of respiration by FCCP was about 2–3-fold. This low value is due to the presence of nigericin [16]; in the absence of nigericin excess FCCP caused a 6–7-fold stimulation of respiration rate in non-phosphorylating mitochondria respiring on succinate at 25°.

Effects of BHA on the proton leak through the mitochondrial inner membrane

If non-phosphorylating mitochondria are titrated with a respiratory inhibitor, a plot of Δp against respiration rate can be constructed. Such a plot indicates the dependence of the proton leak through the mitochondrial inner membrane on Δp. This is because multiplying the respiration rate by the

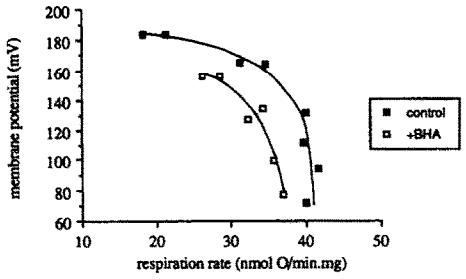

Fig. 1. The effect of BHA on the dependence of membrane potential upon respiration rate during an uncoupler titration of non-phosphorylating mitochondria. Mitochondrial respiration rates and $\Delta\psi$ were measured simultaneously. For this experiment oligomycin was present. Two minutes after the addition of succinate 24 nM FCCP was added and this was followed by two further 24 nM FCCP additions at 1 min intervals. The data shown here are from two separate titrations in the presence of BHA (100 μM) and from two separate control titrations. This experiment was repeated on three separate mitochondrial preparations which gave results similar to those shown here. The maximum respiration rate in the presence of BHA was 95.4 ± 0.9% of the maximum respiration rate for the control incubations (mean of experiments on three separate mitochondrial preparations; $P < 0.05$). All other experimental details are described in the text.

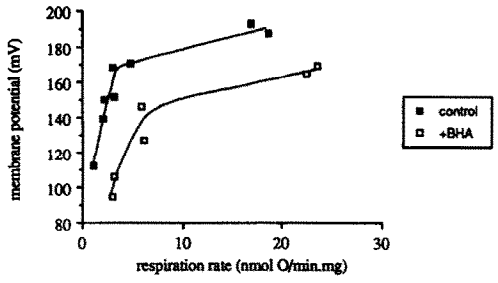

Fig. 2. The effect of BHA on the dependence of membrane potential upon respiration rate during an inhibitor titration of non-phosphorylating mitochondria. Mitochondrial respiration rates and $\Delta\psi$ were measured simultaneously. For this experiment hexokinase, oligomycin and ADP were present. The same results were found in the absence of ADP and hexokinase. Two minutes after the addition of succinate, 1 mM malonate (K^+ salt) was added and was followed at 2 min intervals by two further identical malonate additions. Two minutes after the final malonate addition, FCCP (0.33 μM) was added to return the TPMP electrode trace to its baseline. The data shown here are two separate titrations in the presence of BHA (100 μM) and two separate control titrations. This experiment was repeated on three different mitochondrial preparations with the same results. The uninhibited respiration rate in the presence of BHA was 109.1 ± 1.3% of the uninhibited respiration rate for control incubations (mean of experiments on three separate mitochondrial preparations; $P < 0.02$). All other experimental details are described in the text.

appropriate stoichiometry of proton pumping by the electron transport chain transforms the plot into one of proton efflux as a function of Δp. In a steady state the proton efflux must equal the proton leak, therefore the plot is effectively one of the proton leak as function of Δp. This assumes no slip in the proton pumps [7]. If this assumption is not made, the plot is then one of Δp against the respiration rate balancing the proton leak [7]. It is still a description, albeit less quantitative, of the proton leak as a function of Δp. This eliminates any secondary effect on leak due to alterations in the value of Δp. In Fig. 2, titrations with the respiratory inhibitor malonate are shown in the presence of BHA or control. It can be seen that BHA shifts this curve downwards and to the right. Therefore, at any given value of $\Delta\psi$, the respiration will be greater in the presence of BHA. This is because BHA increases the proton leak through the mitochondrial inner membrane. Therefore the respiration rate increases to compensate for the increased proton leak through the membrane.

Effects of BHA on the phosphorylation system

A titration of phosphorylating mitochondria with a respiratory inhibitor will produce a plot of Δp against respiration rate. Such plots can be easily transformed into the proton current driving the phosphorylation system as a function of Δp. This is possible because the proton leak through the mitochondrial inner membrane is strongly dependent on Δp [22]. At the low values of Δp which are found during phosphorylation the proton leak will be negligible. The respiration rate measured during the titration is largely that required to balance the influx of protons through the phosphorylation system [7]. Therefore a titration of phosphorylating mitochondria with a respiratory inhibitor produces a plot showing the kinetic dependence of the phosphorylation system on Δp. This eliminates any secondary kinetic effects of BHA on phosphorylation via alterations in Δp. In Fig. 3, titrations of phosphorylating mitochondria in the presence of BHA or control are shown. The relatively low rates of state 3 respiration are caused by nigericin [16]. BHA moves this curve down and to the right. Such a shift in the titration implies that there has been a stimulation of the phosphorylation system [7, 10]. However, in these experiments BHA will also increase the respiration rate at a given value of $\Delta\psi$ because it increases the leak of protons through the mitochondrial inner membrane. This increase in proton leak will also displace the titration downwards and to the right. Therefore, the increase in proton leak brought about by BHA must be corrected for before concluding that BHA stimulates the phosphorylation system. To correct for the effect of BHA on the proton leak through the inner membrane the "extra" respiration, balancing the proton leak due to BHA, must be estimated and subtracted from the BHA titration shown in Fig. 3. The data presented in Fig. 2 were used to estimate the "extra" respiration rate. The control respiration rate was subtracted from the rate in the presence of BHA at a series of values of $\Delta\psi$ (150, 160 and 170 mV). This difference in respiration rate was the "extra" respiration required to balance the proton leak due

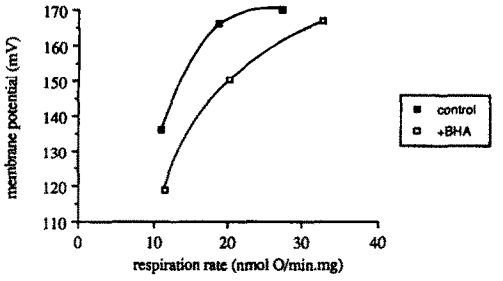

Fig. 3. The effect of BHA on the dependence of membrane potential upon respiration rate during an inhibitor titration of phosphorylating mitochondria. Mitochondrial respiration rates and $\Delta\psi$ were measured simultaneously. For this experiment hexokinase, ADP and Ap_5A were present. For the data shown here each point represents a separate incubation either in the presence of absence of BHA and with malonate (K^+ salt) present from the start of the incubation at concentrations of 0, 0.25 or 0.75 mM, in order of decreasing respiration rate. Succinate was added to give a steady state respiration rate and $\Delta\psi$. Four minutes after the addition of succinate, phosphorylation was stopped by the addition of oligomycin and after this the addition of FCCP (0.33 μM) returned the TPMP electrode trace to its baseline. Samples were then taken for analysis of glucose-6-phosphate as described in the text. This experiment was repeated on two separate mitochondrial preparations with similar results. Essentially the same experiment was performed by sequential addition of inhibitor to give a single titration per incubation. This gave similar results to those shown in this experiment and was repeated on two further mitochondrial preparations. The uninhibited respiration rate in the presence of BHA was 92.1 ± 0.7% of the uninhibited respiration rate for control incubations (mean of experiments on three separate mitochondrial preparations; $P < 0.01$). All other experimental details are described in the text.

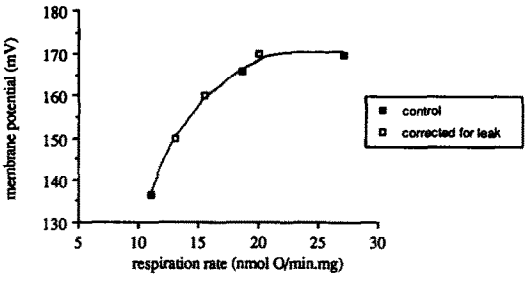

Fig. 4. The effect of BHA on the dependence of membrane potential upon respiration rate for phosphorylating mitochondria corrected for proton leak. The data shown here are from the experiment shown in Fig. 3 in the presence of BHA. The correction for the proton leak due to BHA was carried out as described in the text. This correction procedure was repeated on two further experiments similar to the one outlined in the legend to Fig. 3 and was also done on titrations carried out on a single mitochondrial incubation in the presence of BHA as described in the legend to Fig. 3. All of these gave results similar to those shown here. All other experimental details are described in the text.

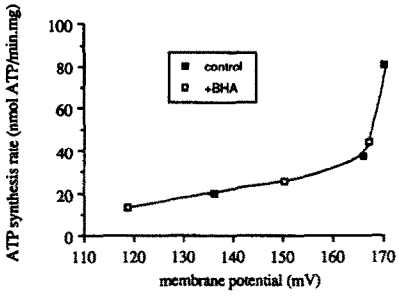

Fig. 5. The effect of BHA on the dependence of the rate of ATP synthesis on the membrane potential. These data were obtained from the mitochondria used in the experiment described in Fig. 3. Different steady state values of $\Delta\psi$ were established by incubating with different concentrations of the inhibitor malonate as outlined in the legend to Fig. 3. After the incubation samples were assayed for glucose-6-phosphate. The rates of ATP synthesis were determined as described in the text and plotted against $\Delta\psi$. In the presence of BHA or control. This experiment was repeated on two separate mitochondrial preparations with similar results. All other experimental details are described in the text.

to BHA at a given value of $\Delta\psi$ and it varied from 12 nmol O/min/mg at 170 mV to 6 nmol O/min/mg at 150 mV. The "extra" respiration was then subtracted from the respiration rates of phosphorylating mitochondria in the presence of BHA at membrane potentials of 150–170 mV. These respiration rates were determined by interpolation of the titration in the presence of BHA shown in Fig. 3. This gave corrected values of respiration which are the rates required to drive phosphorylation and balance the small amount of endogenous proton leak. In Fig. 4, these corrected values are replotted together with the control titration from Fig. 3. When the inhibitor titration from Fig. 3 was corrected for the extra leak due to BHA the apparent stimulation of the phosphorylation system seen in Fig. 3 disappeared (Fig. 4).

In the titration curve in Fig. 2, used to estimate the "extra" respiration for the correction procedure, $\Delta\psi$ increases only slightly as the respiration rate increases. Therefore the amount of extra respiration at a given value of $\Delta\psi$ is difficult to estimate accurately and the correction procedure used in Fig. 4 is susceptible to experimental error. To confirm the conclusions drawn from Fig. 4 the effect of BHA on ATP synthesis by an alternative method was determined. This was done by measuring the rate of synthesis of glucose-6-phosphate as a function of $\Delta\psi$. The data shown in Fig. 5 were from the same experiment used to construct Figs 3 and 4. In this case samples were taken at the end of the experiment to measure the amount of glucose-6-phosphate synthesized. From this the rate of ATP synthesis as a function of $\Delta\psi$ could be calculated. The curves in Fig. 5 show the typical strong dependence of ATP synthesis on $\Delta\psi$ [23–25]. The rates of ATP synthesis were slightly lower here due to the presence of

nigericin; this parallels the effect of the ionophore on respiration rate. From Fig. 5 it can be seen that ATP synthesis rates against $\Delta\psi$ are superimposable in the presence of BHA or control. This corroborates the data shown in Fig. 4 and is consistent with the results of Ferreira [6].

DISCUSSION

This work demonstrates that BHA has two separate effects on mitochondrial oxidative phosphorylation. It inhibits respiration and it increases the proton leak through the mitochondrial inner membrane. BHA has no effect on the mitochondrial phosphorylation system. These results confirm and extend the earlier work of Ferreira [6]. BHA is toxic at high concentrations for two reasons: it depletes cellular ATP by inhibiting respiration and by uncoupling oxidation from phosphorylation. We have also shown that the approach developed by Brand [7] to determine the site of action of hormones on mitochondria can be adapted to investigate the *in vitro* effects of compounds on mitochondria. With this technique it is possible to distinguish the sites of action of a pleiotypic effector on a system such as oxidative phosphorylation. It was therefore particularly informative to utilize this approach to study the effects of BHA on oxidative phosphorylation because BHA has two separate effects on mitochondria. Using this approach we separated out these two effects; this is the first demonstration that the techniques developed by Brand [7] can be easily adapted to study the effects of compounds on mitochondrial oxidative phosphorylation *in vitro* and clearly demonstrates the power of this technique. This approach can be easily extended to other bioenergetic systems and, in addition, it can be used to determine the interaction(s) of compounds of pharmacological interest with mitochondria.

The inhibition of the Δp producers by BHA could result from an interaction with the dicarboxylate carrier or with any component of the electron transport chain. BHA inhibits NADH:ubiquinone oxidoreductase and the cytochome bc_1 complex [6]. Many phenolic compounds, which are similar in structure to BHA, inhibit the cytochrome bc_1 complex [26]. BHA may inhibit respiration through similar interactions with the cytochrome bc_1 complex and NADH:ubiquinone oxidoreductase. A further kinetic analysis of the effects of BHA on the electron flux through individual electron transport chain components is required to definitively establish the site of inhibition by BHA. This would be done in the way used to demonstrate the effects of glucagon on mitochondria [8]. BHA may disrupt membranes [27, 28]. Therefore the inhibition of respiration by BHA may be in part due to disruption of the mitochondrial inner membrane, and not solely to a specific interaction with the electron transport chain. If this is the case, then the absence of an effect of BHA on the phosphorylation system indicates that the activity of the F_0F_1-ATPase and the adenine nucleotide translocase are less sensitive to membrane disruption than is the activity of the electron transport chain.

BHA could increase the proton leak through the mitochondrial inner membrane by acting as a proton shuttle or by disrupting the structure of the membrane [22]. Many other phenolic compounds are proton shuttles [29] and therefore it is probable that a large part of the effect of BHA on the proton leak can be explained by this mechanism. However, BHA also alters membrane structure [27, 28]. These alterations in membrane structure may increase the membrane's proton conductance and thus increase proton leak. It is not possible to distinguish between these two alternatives at present. In conclusion, we have used the technique of Brand [7] to determine how a compound with multiple effects on mitochondria interacts with oxidative phosphorylation. This technique was successful in indicating the disparate effects of BHA on mitochondria and therefore may be of general utility in investigating how pharmacological compounds interact with complex systems such as mitochondria.

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