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Mitochondrial uncouplers with extraordinary dynamic range

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Running title: Mitochondrial uncouplers with extraordinary dynamic range

SYNOPSIS

We have discovered that some weak uncouplers (typified by butylated hydroxytoluene) have a dynamic range of more than 10^6 in vitro: the concentration giving measurable uncoupling is less than one millionth of the concentration causing full uncoupling. They achieve this through a high affinity interaction with the mitochondrial adenine nucleotide translocase that causes significant but limited uncoupling at extremely low uncoupler concentrations, together with more conventional uncoupling at much higher concentrations. Uncoupling at the translocase is not by a conventional weak acid/anion cycling mechanism since it is also caused by substituted triphenylphosphonium molecules, which are not anionic and cannot protonate. Covalent attachment of the uncoupler to a mitochondrially targeted hydrophobic cation sensitises it to membrane potential, giving a small additional effect. The wide dynamic range of these uncouplers in isolated mitochondria and intact cells reveals a novel allosteric activation of proton transport through the adenine nucleotide translocase and provides a promising starting point for designing safer uncouplers for obesity therapy.

Keywords:
Dinitrophenol (DNP), butylated hydroxytoluene (BHT), adenine nucleotide translocase (ANT), uncoupling, obesity, carboxyatractylate (CAT)

INTRODUCTION

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Weight gain, leading to obesity, occurs when energy intake consistently exceeds energy expenditure. In principle, obesity can be treated by reducing caloric intake, by increasing expenditure, or by both approaches simultaneously. Current pharmacological therapies using sibutramine or orlistat [1] or, more recently, rimonabant [2] act primarily to reduce energy intake, and energy expenditure has been relatively neglected as a target for obesity therapy [3]. One way to increase energy expenditure is to use uncouplers to weaken the coupling between fuel oxidation and ATP production. Uncouplers work by transporting protons across the mitochondrial inner membrane, short-circuiting the normal pathway of oxidative ATP synthesis driven by proton flow and causing the loss of calories as heat. They are usually lipophilic weak acids that pick up a proton, diffuse across the mitochondrial inner membrane into the matrix, deprotonate and then exit as anions before repeating the catalytic cycle.

The notable success of the uncoupler 2,4-dinitrophenol (DNP) as a treatment for human obesity in the 1930s provided an important proof of concept and showed that the beneficial effect of uncoupling on energy expenditure is not overwhelmed by compensatory increases in caloric intake. The early literature on treatment of humans and recent studies in rats suggest that dinitrophenol matches or outperforms modern drug candidates at causing weight loss [1, 4-6]. However, the narrow therapeutic window of dinitrophenol and other conventional uncouplers led to the abandonment of their official use in treatment of obesity. Threefold to tenfold increases above the minimum effective dose result in too much uncoupling, leading to compromised ATP production, hyperthermia and death. Concern over this narrow therapeutic window was one of the primary reasons that dinitrophenol was withdrawn from the market in 1938 [1, 5-7].

For uncouplers to work safely, they should cause uncoupling that increases very little as their concentration rises, potentially widening the difference between therapeutic and toxic doses and giving a wide therapeutic window. Knowing which features of uncouplers to manipulate to give the desired wide dynamic range (ratio of concentrations giving maximum and minimum observable uncoupling) is crucial to the rational design of uncouplers that may be safe for use in obesity treatment. We present here uncouplers with an extraordinarily wide dynamic range of greater than 10^6-fold, and analyze the mechanisms by which this is achieved.

MATERIALS AND METHODS

Mitochondria

Rat liver mitochondria were isolated from female Wistar rats (4-8 weeks old) in medium containing 0.25 M sucrose, 5 mM Tris-HCl, and 2 mM EGTA (pH 7.4 at 4°C) [8]. Rat skeletal muscle was dissected from the hind limbs and placed in ice-cold medium containing 100 mM KCl, 2 mM EGTA and 50 mM Tris-HCl. Skeletal muscle mitochondria were isolated at 4°C as previously described [9]. Mitochondrial protein was assayed using the biuret method, with bovine serum albumin as standard.

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Rat thymocytes

Thymocytes were isolated from the thymus of female Wistar rats (4-6 weeks old) as previously described [10]. Cells were stored for up to 4 h in plastic flasks at 37°C and regularly gassed with CO_2/air (1:19). Cell concentration was determined using a haemocytometer. The viability of freshly isolated cells was greater than 95% as determined by Trypan blue exclusion. Isolation and incubations were in RPMI medium (supplemented with 2 mM glutamine and without glucose).

Oxygen consumption

Mitochondria (0.5 mg protein/ml) were incubated at 37°C in an oxygen electrode (Rank Brothers, UK) connected to a Powerlab data analysis system (Powerlab Chart 5, ADInstruments) in "reaction medium" containing 120 mM KCl, 5 mM KH_2PO_4, 3 mM Hepes, 1 mM EGTA, pH 7.2, supplemented with 5 µM rotenone, 1 µg/ml oligomycin, and 80 ng/ml nigericin. Uncouplers were added in 1 µl of dimethylsulphoxide using DMSO stocks prepared at different uncoupler concentrations. Where appropriate, malonate was present at concentrations from 0 to 2 mM to give different membrane potentials. Respiration was initiated by adding 4 mM succinate. At the end of the run, 0.3 µM FCCP was added to uncouple the mitochondria fully. Reaction volume was 3.5 ml (1 ml when membrane potential was measured in parallel using radiolabelled TPMP). To eliminate any problems of carry-over of uncouplers, we always checked that titrations from high to low concentrations gave the same results as titration from low to high concentrations.

Thymocyte respiration was measured in the same system for 10 min at 37°C in 1 ml RPMI medium in the presence of 160 ng/ml oligomycin and uncoupler (from DMSO stock). Oligomycin was absent in the experiments using gramicidin (Fig. 6F,G).

Membrane potential

Mitochondria (0.5 mg protein in 1 ml) were incubated in microcentrifuge tubes containing reaction medium supplemented with 1 µM $TPMP^+$, 0.2 µCi/ml ^{3}[H]$TPMP^+$ (American Radiolabeled Chemicals, St. Louis, MO), 4 mM succinate, and uncoupler (from DMSO stock) at 37°C for 2 min. Where appropriate, malonate was present at concentrations from 0 to 2 mM to give different membrane potentials. The mitochondria were pelleted by centrifugation and ^{3}[H]$TPMP^+$ in the pellet and supernatant was determined by scintillation counting [11]. Membrane potential was calculated assuming a mitochondrial volume of 0.8 µl/mg protein and a TPMP binding correction of 0.4 [11]. Runs were carried out in triplicate.

Novel uncouplers

BHT linked to triphenylphosphonium ("mitoBHT"; (3,5-di-*tert*-butyl-4-hydroxybenzyl)-triphenylphosphonium), and tricyclohexylphosphonium ("cyclohexylmitoBHT"; (3,5-di-*tert*-butyl-4-hydroxybenzyl)-tricyclohexylphosphonium), and compounds 0376 (NNC-0112-0000-0376; 1-

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hydroxy-naphthalene-2-carboxylic acid(4-cyano-2-trifluoromethoxy-phenyl)-amide) and 2604 (NNC-0112-0000-2604; 2-methanesulphonyl-3-(2-methyl-5-nitro-*1H*-indol-3-yl)-acrylonitrile) were prepared according to [12]. MitoDNP and mitoQ were prepared as described in [13-16]

Statistics and regression analysis

Values are given as mean ± SEM or mean ± SD where appropriate. GraphPad Prism (Version 4) was used to find the best-fit linear regression (log-log plots), deduce the slopes and differences between slopes, and determine the significance of differences between means by ANOVA. P values < 0.05 were taken to be significant.

RESULTS

Uncouplers with a wide dynamic range of activity

Fig. 1 gives the structures of the uncouplers used. They are all lipophilic weak acids except for the mitoQ and triphenylphosphonium (TPP) series.

Fig. 2 shows oxygen consumption by isolated rat liver mitochondria. The slow, fully coupled rate occurred in the presence of vehicle (dimethylsulphoxide, DMSO) alone, while the fully uncoupled rate after addition of excess chemical uncoupler (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, FCCP) was about three-fold faster, reflecting normal respiratory control. Fig. 2A shows the result of titrating respiration with a conventional uncoupler, carbonylcyanide *m*-chlorophenylhydrazone (CCCP). The titration gave the conventional result: respiration was markedly stimulated by 10^{-8} M CCCP, and increased in proportion as CCCP concentration was raised fourfold.

2,6-*bis*(1,1-dimethylethyl)-4-methylphenol, more commonly known as butylated hydroxytoluene (BHT), has been reported to be a conventional but weak uncoupler [17-19]. The molecule is lipid-soluble, but the pK of the phenol group is rather high (pK = 10), so uncoupling should be limited by the low concentration of the anionic form. When we titrated respiration with BHT (Fig. 2B), it gave unexpected results. There was marked uncoupling at extremely low concentrations, 2×10^{-12} M BHT, but only very modest increases in uncoupling as the concentration of BHT was raised a million-fold to 2×10^{-6} M.

The unusual characteristics of uncoupling by BHT are clearer when the results are plotted on logarithmic axes. Fig. 2C shows the conventional behaviour exhibited by CCCP: doubling CCCP concentration doubled its uncoupling effect, so the plot of log change in respiration rate against log CCCP concentration gave a straight line with a slope (Table 1) of about 1. Also plotted is the rate at each CCCP concentration after subsequent addition of excess FCCP to fully uncouple, showing that there was no confounding effect on maximum respiratory capacity caused by CCCP addition. The dynamic range of CCCP concentrations from the lowest measured uncoupling to the maximum

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uncoupling was about 10. Rather similar results were obtained with dinitrophenol (Fig. 2D), which gave a slope of about 0.6 and a dynamic range of 10-100. Fig. 2E shows the same plot for BHT. Doubling BHT concentration caused only a small increase in uncoupling, and a straight line fit through the points gave a slope of only 0.1, giving a dynamic range of more than 10^6.

Benzoic acid is another 'weak' uncoupler, with an appropriate pK of 4.2 but low solubility of the anionic form in the membrane, which should limit its ability to uncouple. However, at low concentrations benzoic acid behaved like BHT, with a slope of about 0.1. It failed to give full uncoupling at higher concentrations (Fig. 2F).

Table 1 reports the slopes of these log-log plots for different uncouplers and the concentrations that gave half-maximal uncoupling. CCCP and FCCP gave slopes near 1, corresponding to the expected narrow dynamic range. Dinitrophenol gave an intermediate slope. BHT, benzoic acid, the BHT analogue (1,1-dimethylethyl)-4-methoxyphenol (butylated hydroxyanisole, BHA), and a number of novel uncouplers (mitoBHT, cyclohexylmitoBHT, compound 0376, compound 2604, mitoDNP and mitoQ_{10} - see Fig. 1 for structures) gave very shallow slopes, corresponding to a very wide dynamic range.

To investigate whether uncouplers like BHT also uncouple mitochondria from cells whose energy metabolism might be more relevant in obesity treatment, similar respiration measurements were performed with CCCP, BHT, and benzoic acid in rat skeletal muscle mitochondria (Fig. 3A-C, Table 2). The results were very similar to those observed using liver mitochondria, but the extent of uncoupling in skeletal muscle mitochondria was slightly lower than in liver mitochondria (compare Fig. 2C, E-F with Fig. 3A-C).

Potential-dependence contributes little to the wide dynamic range of uncoupling by BHT

One possible explanation for the wide dynamic range of uncoupling by BHT is potential-dependence of the anion translocation step of the uncoupling cycle. If this step strongly limited uncoupling and was steeply dependent on membrane potential, then addition of more BHT might lower the membrane potential sufficiently to attenuate the uncoupling ability of BHT, leading to the observed weak dependence of uncoupling on BHT concentration.

Fig. 4A shows the kinetic dependence of proton leak rate on membrane potential in liver mitochondria at different concentrations of BHT. The ability of BHT to uncouple by catalyzing proton leak was not unusually dependent on the membrane potential compared to other uncouplers such as fatty acids [20], uncoupling proteins [21, 22] or the basal proton leak pathway catalyzed by the adenine nucleotide carrier [23].

Any effect of potential-dependence of uncoupling by BHT can be eliminated by comparing proton leak rates at a potential common to all uncoupler concentrations. If unusual potential-dependence caused the wide dynamic range of BHT, at a common potential BHT should behave like CCCP with a slope of 1.0 in the log-log plots. However, after correction for potential, the slope for

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BHT remained shallow, with a value of 0.20 (Fig. 4B), albeit slightly steeper than the slope of 0.13 obtained from the same dataset without correcting to a common membrane potential.

Thus, there is a self-limiting effect of decreasing membrane potential on the ability of BHT to uncouple mitochondria, but the effect is small and it contributes little to the wide dynamic range of uncoupling by BHT.

Role of the adenine nucleotide translocase in the uncoupling process

The adenine nucleotide translocase is an abundant inner membrane protein that imports ADP and exports ATP during oxidative phosphorylation. It can also enhance uncoupling of mitochondria by dinitrophenol and several other uncouplers [24-26].

To test whether uncoupling mediated by the adenine nucleotide translocase was involved in the wide dynamic range of BHT, we examined the effects of carboxyatractylate (CAT), a high-affinity inhibitor of the adenine nucleotide translocase (Fig. 5). Fig. 5C shows that CAT fully inhibited BHT-induced uncoupling at BHT concentrations below 10^{-7} M (compare Fig. 2E). It abolished uncoupling induced by benzoic acid (Fig. 5D), but had no effect on uncoupling by CCCP (Fig. 5A) and only a small effect on uncoupling by dinitrophenol (Fig. 5B). The effects of CAT on the slopes of the log-log plots are reported in Table 1. In the presence of CAT, the wide dynamic range of BHT was abolished, and CCCP, dinitrophenol and BHT all gave slopes near 1. These effects of CAT were mimicked by bongkrekic acid, another specific inhibitor of adenine nucleotide translocase (results not shown), confirming that they operate through inhibition of this translocase. Thus the wide dynamic ranges of uncoupling by BHT and benzoic acid are completely dependent on the activity of the adenine nucleotide translocase.

The properties of the CAT-sensitive uncoupling by these compounds, re-examined in linear coordinates, are shown in Fig. 5E-H. Uncoupling through the adenine nucleotide translocase, as indicated by CAT-sensitive uncoupling, appeared to follow simple saturation kinetics. Table 1 presents V_{max} and K_m values for each uncoupler. BHT was a good substrate for the uncoupling activity of the adenine nucleotide translocase, with a K_m in the nM range and a V_{max} giving about 50% increase in respiration rate. The translocase had a very low K_m for benzoic acid, but at V_{max} gave only about 25% increase in respiration, while dinitrophenol was a relatively poor substrate, with higher K_m and lower V_{max} values.

Thus the highly-desirable wide dynamic range of uncoupling by BHT is caused by the overlap of two effects: medium-capacity, high-affinity, CAT-sensitive uncoupling through the adenine nucleotide translocase at low BHT concentrations, and high-capacity, low-affinity, CAT-insensitive uncoupling through other pathways at high BHT concentrations. Surprisingly, CCCP is also a good substrate for the adenine nucleotide translocase, but since (unlike BHT) the K_m for translocase-mediated uncoupling is similar to the $K_{0.5}$ for CAT-independent uncoupling (Table 1), the two effects

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overlap and translocase-catalyzed uncoupling had no discernable effect on the dynamic range for CCCP.

Competition between uncouplers and nucleotides for the adenine nucleotide translocase

To test whether the nucleotide substrates of the adenine nucleotide translocase inhibit uncoupling by BHT, we examined the effect of 1 mM ADP on uncoupling by BHT. ADP lowered the apparent affinity for BHT but, unlike CAT, did not prevent translocase-catalyzed uncoupling (result not shown). Presumably, the nM affinity of BHT for the adenine nucleotide translocase (Table 1) allows it to compete effectively with ADP (K_m (ADP) = 1-10 μM) [27].

Does BHT prevent nucleotide exchange on the translocase? We found that BHT, at concentrations up to 2 x 10^{-5} M, had no significant effect on the state 3 respiration of liver mitochondria (Fig. 6A), suggesting that any inhibition is without functional consequences.

Uncoupling with wide dynamic range in rat thymocytes

To test directly whether the wide dynamic range of uncoupling by BHT could still be observed at cellular concentrations of adenine nucleotides and other physiological metabolites, we repeated the measurements of dynamic range in rat thymocytes. Fig. 6B-E shows that the uncoupling effects of CCCP, dinitrophenol, BHT and benzoic acid in thymocytes were similar to those observed in isolated liver mitochondria, except that BHT did not give full uncoupling in the higher concentration range. The same pattern of slopes in log-log plots and of dynamic range were observed: CCCP had a steep slope and narrow dynamic range; dinitrophenol was similar to CCCP but the slope appeared to be a little shallower; BHT and benzoic acid had very shallow slopes and a much wider dynamic range.

As long as mitochondrial protonmotive force remains sufficiently high to drive ATP synthesis, ATP production in cells will not necessarily be compromised significantly by limited uncoupling of oxidative phosphorylation. This is illustrated by the low control that the proton leak pathway has over ATP production rates in hepatocytes [28] or thymocytes [29]. Nevertheless, we tested empirically whether uncoupling by BHT compromises ATP production in thymocytes, either by lowering protonmotive force too much, or by preventing adequate ATP-ADP exchange on the adenine nucleotide translocase. In the presence of low concentrations of gramicidin to increase the flux of sodium across the plasma membrane, a considerable proportion of respiration is used to make ATP to drive the ouabain-sensitive Na^+/K^+ ATPase, and the ouabain-sensitive respiration rate is a measure of the rate of this ATP-turnover pathway [30, 31]. This is shown in Fig. 6F: concentrations of gramicidin between 0.2 and 1.7 μg/ml doubled thymocyte respiration rate in the absence but not the presence of ouabain, so that under these conditions about half of the thymocyte respiration rate was used to drive the sodium pump. Fig. 6G shows this ouabain-sensitive respiration at various concentrations of BHT. There was no significant effect of up to 10^{-5} M BHT on ATP turnover by the Na^+/K^+ ATPase in thymocytes, showing that even at the highest concentrations used, 10^7-fold higher than doses that gave

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significant uncoupling, there was little or no effect on the ability of thymocytes to make ATP even in response to the extra demand imposed by gramicidin-induced sodium cycling.

Effect of targeting BHT to mitochondria

The therapeutic safety of uncouplers might be enhanced by targeting them to mitochondria by covalent linkage to triphenylphosphonium (TPP) cations; lipophilic molecules that accumulate in mitochondria driven by the membrane potential [32]. In addition, as discussed previously [13], an uncoupler linked to TPP might be strongly self-limiting (because as it uncouples it lowers membrane potential, attenuating further accumulation and therefore attenuating uncoupling), leading to a wider dynamic uncoupling range. Dinitrophenol linked to TPP ("mitoDNP") has been investigated previously [13] but was found to be ineffective at uncoupling.

We investigated the uncoupling properties of BHT covalently linked to triphenylphosphonium ("mitoBHT") or to tricyclohexylphosphonium ("cyclohexylmitoBHT"). Fig. 7A shows that mitoBHT was accumulated about 1500-2000-fold into mitochondria in response to the membrane potential set up by succinate oxidation, and released when that potential was fully dissipated by uncoupling with FCCP, consistent with the expected properties of the mitochondrially-targeted molecule. CyclohexylmitoBHT was taken up and released in the same way by mitochondria (not shown). Fig. 7B shows that, like BHT, mitoBHT is an effective uncoupler, with a dynamic range of at least 10^9. Fig. 7C shows the log-log plot of increase in respiration rate against mitoBHT concentration; the slope was 0.06 (Table 1), similar to or even less than the slope for BHT. However, mitoBHT uncoupled at even lower concentrations than BHT, with a $K_{0.5}$ of 6 nM for mitoBHT, compared to 60 nM for BHT (Table 1). As a control, methyltriphenylphosphonium (TPMP: triphenylmethylphosphonium; mitoBHT with BHT replaced by a methyl group) caused much less uncoupling over the same range of concentrations. CyclohexylmitoBHT had similar properties to mitoBHT (Table 1). MitoBHT also uncoupled skeletal muscle mitochondria at very low concentrations (Fig. 3D). As with BHT, the extent of this uncoupling was slightly lower in muscle mitochondria than in liver mitochondria (compare Fig. 3D with Fig. 7C).

In the presence of CAT, stimulation of liver mitochondrial respiration rates was inhibited at low mitoBHT concentrations (Fig. 7D), indicating that, as with BHT, most of the wide dynamic range for mitoBHT was caused by interactions with the adenine nucleotide translocase at low uncoupler concentrations. However, unlike BHT, the slope of the log-log plot was not brought to 1 by CAT, but only to about 0.4 (Table 1). The CAT-sensitive component showed simple saturation kinetics (Fig. 7E) with a nM K_m (Table 1).

We tested whether the introduction of the TPP group had rendered mitoBHT more potential-sensitive than BHT, causing the slope of the log-log plot to be less than 1.0 even in the presence of CAT. Mitochondrial proton leak rate in the presence of CAT was compared at a membrane potential common to all mitoBHT concentrations (Fig. 7F). After this correction for any effects of membrane

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potential, the slope was increased from 0.4 to about 0.7 (Fig. 7G), suggesting that self-limitation of uncoupling through attenuation of membrane potential was a factor in the wide dynamic range of mitoBHT, but much less important than the interaction with the adenine nucleotide translocase.

MitoBHT uncoupled respiration in thymocytes much as it did in mitochondria (Fig. 7H). The slope in log-log plots was shallow (0.1) and the dynamic range was once again very large, more than 10^6.

Effects of other mitochondrially-targeted uncouplers

To investigate why mitoDNP was apparently ineffective even though mitoBHT worked well, we reinvestigated uncoupling by mitoDNP. We found that it behaved in the same way as mitoBHT, but with much lower V_{max} at the translocase (Fig. 7C), explaining why the uncoupling effect was missed previously [13].

$MitoQ_{10}$, a derivative of coenzyme Q [14-16], also behaved like mitoBHT, but with a lower V_{max} (Fig. 8A). Interestingly, decylTPP showed similar behaviour (Fig. 8B), even though it lacks a protonatable group. Uncoupling by both $mitoQ_{10}$ and decylTPP at concentrations below 10^{-7}M was fully inhibited by CAT (Fig. 8A, B), showing that uncoupling at these concentrations required translocase activity. Similarly, the slopes of the log-log plots for $mitoQ_{10}$ and decylTPP, were steeper in the presence of CAT (see Table 1). Since the uncoupling activity of the mitoQ compounds was similar to that of the TPP series, but the antioxidant effects of the mitoQ compounds are much greater than those of the TPP series [14-16], the antioxidant effect of mitoQ must be distinct from the uncoupling mechanism through the translocase revealed here.

While mitoBHT, at 10^{-9} M, increased state 4 respiration by about 50%, the mitoQ and TPP series of compounds increased it by only 10-20% (Fig. 8C, D). Since TPP^+ (and mitoQ) compounds cannot deprotonate/reprotonate, the uncoupling mechanism for these compounds is not via weak acid cycling. Presumably it is by allosteric activation of a proton leak pathway through the adenine nucleotide translocase. The greater uncoupling by mitoBHT compared to mitoQ and TPP analogues suggests that there are structural constraints for this allosteric activation.

DISCUSSION

Mitochondrial uncoupling is an effective way to reduce body weight in humans, but using uncouplers such as dinitrophenol is problematic because the toxic dose is close to the therapeutic dose. The problem is the steep concentration-dependence of uncoupling by dinitrophenol and other conventional uncouplers. Unlike dinitrophenol, BHT partially uncoupled mitochondria and cells at extremely low concentrations. This uncoupling was only slightly dependent on concentration, leading to the extraordinarily wide dynamic range of uncoupling by BHT.

Our experiments show that the main reason for this wide dynamic range is the overlap of two effects as shown in Fig. 9: (i) medium-capacity, high-affinity, CAT-sensitive uncoupling through the

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adenine nucleotide translocase at low BHT concentrations and (ii) high-capacity, low-affinity, CAT-insensitive uncoupling through other pathways at high BHT concentrations. Uncouplers such as CCCP and DNP, in which translocase-mediated uncoupling is minor (or has a K_m similar to the $K_{0.5}$ for CAT-insensitive uncoupling), show steep dependence of uncoupling on concentration. Conversely, uncouplers such as BHT, with high translocase-catalyzed rates and high affinities relative to the CAT-insensitive ones, show wide dynamic range and pseudo-linear shallow-slope concentration dependence in log-log plots. Benzoic acid uncouples only through the adenine nucleotide translocase, explaining its ability to cause only mild uncoupling over a wide range of concentrations. Differences in the involvement of the adenine nucleotide translocase in the uncoupling mechanism explains the varying concentration-dependence of uncoupling – the bigger the involvement of the translocase, the less the uncoupling effect depends on uncoupler concentration.

How well do the uncouplers work in mitochondria from tissues other than liver? A comparison revealed slightly lower effects in skeletal muscle mitochondria (Fig. 3) than in liver mitochondria (Fig. 2). ANT1 is the predominant isoform in skeletal muscle whereas ANT2 predominates in liver [33]. Differences in the affinities of ANT isoforms for these uncouplers might explain this difference in uncoupling effect. Despite this, uncouplers like BHT or mitoBHT still showed the desirable wide dynamic range of activity in mitochondria from both tissues. This wide dynamic range was also observed at the cellular level using thymocytes, suggesting that there is no major tissue specificity.

At first sight it may appear unlikely that compounds like BHT could uncouple through the adenine nucleotide translocase in cells, where concentrations of adenine nucleotides are high and might be expected to prevent their action. However, the K_m of the adenine nucleotide translocase for BHT and other wide dynamic range uncouplers is remarkably low ($K_m < 10^{-8}$ M), so they can uncouple mitochondria at very low concentrations even in the presence of mM ADP or ATP (K_m (ADP) = 1-10 μM; K_m (ATP) = 1-150 μM [27]. This is apparent from our measurements of the competition by nucleotides in isolated mitochondria, and from the robust effect of these compounds at low concentrations in cells (Fig. 6; Fig. 7H).

The mechanism by which molecules activate uncoupling through the adenine nucleotide translocase is not known. Previous workers have suggested that the translocase enhances uncoupling by transporting the anionic form of the uncoupler across the inner membrane, catalyzing a step that otherwise significantly limits the cycling rate [24-26]. However, we find that CAT-sensitive uncoupling can be activated by mitoBHT and cyclohexylmitoBHT (which are zwitterionic when deprotonated, so should not be driven out through the translocase by the membrane potential). Most importantly, we find CAT-sensitive uncoupling by compounds that cannot uncouple directly via the weak acid cycling mechanism because they have no anionic form and cannot protonate and deprotonate as required for this mechanism: the substituted TPP cations (Fig. 8). We therefore prefer a model in which most or all of these hydrophobic CAT-sensitive uncouplers bind to the adenine nucleotide translocase and allosterically induce net proton transport. Perhaps they do this through the

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same pathway by which AMP induces proton transport through the translocase [34], and as alkylsulphonates and perhaps fatty acids do in the uncoupling protein family [35, 36].

As well as uncoupling, BHT and related compounds like butylated hydroxyanisole or diisopropylphenol are reported to inhibit electron transport or ATP synthesis [18, 19, 37, 38]. However, these secondary effects are seen only at relatively high concentrations, 10^{-3} to 10^{-5} M. In isolated mitochondria, there was no inhibition of electron transport capacity at the BHT concentrations we have used (below 10^{-5} M) when the mitochondria were fully uncoupled by FCCP (Fig. 2E,F; Fig. 5C,D; Fig. 7C,D), and no inhibition of oxidative phosphorylation. In thymocytes, BHT did not uncouple fully at the higher concentrations (Fig. 6D), perhaps indicating secondary inhibition, but this problem was not observed with mitoBHT (Fig. 7H). However, 10^{-5} M BHT did not significantly inhibit the ability of thymocytes to produce ATP for the sodium pump (Fig. 6G), suggesting that even at this concentration, 10^{7}-fold higher than the BHT concentration that gives significant uncoupling in thymocytes, any secondary effects of BHT on electron transport or ATP synthesis had only minor bioenergetic consequences.

Like BHT, mitoBHT had a wide dynamic range of uncoupling activity, but it caused half-maximal uncoupling at even lower concentrations. This desirable property may be caused partly by potential-sensitive uncoupling, since the concentration-dependence of translocase-independent uncoupling became less steep when the effects of membrane potential were removed (Fig. 7G), or simply by greater mitochondrial accumulation because of that potential. It may also be related to differences in hydrophobicity and partitioning in the membrane or binding to the allosteric site on the adenine nucleotide translocase. Whatever the reasons, the low $K_{0.5}$, wide dynamic range, relatively large effect and predicted mitochondrial targeting in cells make mitoBHT an attractive candidate for further development.

The wide dynamic range of uncoupling by BHT and benzoic acid provides an excellent starting point for the design of novel uncouplers that could be used to modulate the burning of calories in humans for the treatment of obesity. The production of reactive oxygen species decreases strongly when mitochondrial protonmotive force is lowered even slightly by uncoupling [39-41]. These uncouplers therefore also present an alternative approach to decreasing radical generation and perhaps treating age-related disorders, particularly since BHT and mitoBHT have antioxidant as well as uncoupling properties. Several improvements could be made to their ability to uncouple. Their properties could be altered to raise or lower the V_{max} of their uncoupling through the adenine nucleotide translocase to fine-tune this component, or to enhance the binding of the uncoupler to the translocase to give even higher specificity. The properties of benzoic acid that allow it to uncouple only through the translocase could be exploited, and it could be used as a starting compound for the design of molecules that have the desired affinity and V_{max} for the translocase with no further uncoupling at higher concentrations. More generally, our studies reinforce the importance of the

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adenine nucleotide translocase as an important drug target for the identification of molecules that can alter the efficiency and rate of energy expenditure [34].

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FOOTNOTES

The abbreviations used are: ANT, adenine nucleotide translocase; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CAT, carboxyatractylate; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DMSO, dimethylsulphoxide; DNP, dinitrophenol; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; TPP, triphenylphosphonium.

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Table 1. Effect of different uncouplers on the respiration of rat liver mitochondria

Total action: data from Fig. 2C-F, Fig. 7C, Fig. 8A-B, and similar experiments. $K_{0.5}$ values were calculated from the linear regressions. Action not through ANT (adenine nucleotide translocase): uncoupling insensitive to CAT, data from Fig. 5A-D, Fig. 7D, Fig. 8A-B, and similar experiments. Action through ANT: uncoupling sensitive to CAT, data from Fig. 5E-H, Fig. 7E, and similar experiments. K_m and V_{max} values were calculated from hyperbolic regressions using GraphPad Prism 4.0. Data from experiments not shown in the Figs are means ± SEM of four independent experiments.

Uncoupler	Total action		Action not through ANT	Action through ANT	
	Slope in log/log plot	$K_{0.5}$ (M)	Slope in log/log plot	V_{max} (change in respiration rate, % of DMSO control)	K_m (M)
CCCP	0.92 ± 0.06	2 (±0.4) x 10^{-8}	0.95 ± 0.11	46 ± 41	3 (±5) x 10^{-8}
FCCP	0.71 ± 0.07	3 (±0.8) x 10^{-9}	---	---	---
DNP	0.55 ± 0.11	4 (±2) x 10^{-6}	0.90 ± 0.11	19 ± 12	2 (±10) x 10^{-7}
BHT	0.11 ± 0.02	6 (±1) x 10^{-8}	0.93 ± 0.22	55 ± 6	2 (±1) x 10^{-9}
BHA	0.15 ± 0.03	5 (±1) x 10^{-8}	---	---	---
Benzoic acid	0.06 ± 0.02	---	---	28 ± 3	3 (±5) x 10^{-13}
MitoBHT	0.06 ± 0.02	6 (±2) x 10^{-9}	0.38 ± 0.03	47 ± 9	8 (±14) x 10^{-10}
Cyclohexyl-mitoBHT	0.05 ± 0.01	4 (±1) x 10^{-8}	---	---	---
0376	0.05 ± 0.01	2 (±1) x 10^{-5}	---	---	---
2604	0.09 ± 0.02	8 (±1) x 10^{-9}	---	---	---
MitoDNP	0.08 ± 0.03	---	---	---	---
$MitoQ_{10}$	0.08 ± 0.03	---	0.39 ± 0.08	---	---
DecylTPP	0.23 ± 0.07	5 (±3) x 10^{-6}	0.54 ± 0.1	---	---

Table 2. Effect of different uncouplers on the respiration of rat skeletal muscle mitochondria

Data from experiments of Fig. 3. $K_{0.5}$ values were calculated from the linear regressions using GraphPad Prism 4.0. Data are means ± SEM of three to four independent experiments.

Uncoupler	Slope in log/log plot	$K_{0.5}$ (M)
CCCP	1.10 ± 0.18	2 (±1) x 10^{-7}
BHT	0.10 ± 0.02	---
Benzoic acid	0.01 ± 0.05	---
MitoBHT	0.15 ± 0.03	7 (±3) x 10^{-5}

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FIGURE LEGENDS:

Figure 1. Structures of compounds used.
"n" denotes the number of alkyl chain carbon atoms. tBu: tertiary butyl.

Figure 2. Effect of different uncouplers on the respiration of rat liver mitochondria.
(A and B) Representative traces of oxygen consumption in the presence of (A) CCCP or (B) BHT. Succinate (4 mM) was added to initiate respiration (arrowhead). DMSO, different concentrations of CCCP or BHT or excess (0.3 μM) FCCP were present where indicated. **(C-F)** Double logarithmic plots of uncoupling. Open or grey circles: titration of respiration with CCCP, dinitrophenol, BHT, or benzoic acid. Closed circles: rate at each uncoupler concentration after subsequent addition of 0.3 μM FCCP, giving the maximum respiratory capacity of the mitochondria. Data are means ± SEM of four independent experiments. Lines were fitted by regression (to the grey points in (F)).

Figure 3. Effect of different uncouplers on the respiration of rat skeletal muscle mitochondria.
Double logarithmic plots of uncoupling in skeletal muscle mitochondria by **(A)**.CCCP, **(B)** BHT, **(C)** benzoic acid, and **(D)** mitoBHT. Open circles: titration of respiration with CCCP, BHT, benzoic acid or mitoBHT. Closed circles: rate at each uncoupler concentration after subsequent addition of 0.3 μM FCCP, giving the maximum respiratory capacity of the mitochondria. Data are means ± SEM of four independent experiments. Lines were fitted by regression.

Figure 4. Effect of membrane potential on uncoupling of rat liver mitochondria by BHT.
(A) Kinetic response of proton leak rate to membrane potential in the presence of DMSO or different concentrations of BHT as indicated. Proton leak rate was measured as the respiration rate used to drive it; membrane potential was varied by adding different concentrations of malonate. Data are means ± SEM of four independent experiments. **(B)** Uncoupling by BHT. Data taken from (A) at the highest common membrane potential of 164.3 mV (solid line; slope = 0.20 ± 0.02; error bars for interpolated points are the weighted means of the error bars on flanking experimental points), or at the highest potential for each uncoupler concentration (dashed line; slope = 0.13 ± 0.04; points and error bars as in (A) but omitted here for clarity). The slopes are significantly different; $P < 0.05$.

Figure 5. Effect of different uncouplers on the respiration of rat liver mitochondria in the presence of carboxyatractylate (CAT).
(A-D) Double logarithmic plots of uncoupling in the presence of 2 nmol CAT/mg protein (added before succinate). Open circles: titration of respiration with CCCP, dinitrophenol, BHT, or benzoic acid. Closed circles: rate at each uncoupler concentration after subsequent addition of 0.3 μM FCCP. Data are means ± SEM of four independent experiments. Lines were fitted by regression. **(E-H)** CAT-

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sensitive stimulation of mitochondrial respiration rate in linear coordinates. CAT-sensitive stimulation of respiration rate (open circles; shaded profile; line fitted as a rectangular hyperbola) was estimated as total uncoupling (filled circles; data from Fig. 2C-F where CAT was absent; line fitted as the sum of the other two lines) minus CAT-insensitive respiration rates (open triangles; data from Fig. 5A-D where CAT was present; linear fit).

Figure 6. Effect of different uncouplers on mitochondrial ATP synthesis and the respiration of rat thymocytes.

(A) State 3 respiration in rat liver mitochondria with DMSO (vehicle) and at different BHT concentrations in the presence of 50 μM p^1,p^5-di(adenosine-5')pentaphosphate (an adenylate kinase inhibitor). State 3 respiration rates were measured after the addition of succinate and 0.1 mM ADP. State 3 rates without DMSO are included for comparison. Data are means ± SD of two independent experiments. **(B-E)** Double logarithmic plots of uncoupling by CCCP, dinitrophenol, BHT, or benzoic acid. Data are means ± SEM of three or four independent experiments. Lines were fitted by regression. The slopes are (B) 1.07 ± 0.15, (C) 0.78 ± 0.20, (D) 0.11 ± 0.07, and (E) 0.10 ± 0.04. For BHT and benzoic acid, lines were fitted only between 10^{-12} and 10^{-9} M (shaded diamonds). **(F)** Gramicidin titration of thymocyte respiration. Gramicidin was sequentially added to the cells after a steady baseline was achieved in the presence (filled squares) or absence (open squares) of 1 mM ouabain. Data are means ± SEM of four experiments. **(G)** Ouabain-sensitive respiration driving the Na^+/K^+ ATPase at different BHT concentrations. Gramicidin at 0.57 μg/ml and ouabain at 1 mM were added before BHT, and cellular respiration rate was measured for 10 min. Basal cell respiration rates with and without ouabain are included for comparison. Data are means ± SEM of three to five independent experiments. Rates and differences were not significantly affected by BHT (ANOVA).

Figure 7. Uncoupling effects of mitoBHT in rat liver mitochondria and rat thymocytes.

(A) Uptake and release of mitoBHT. The electrode was calibrated by five additions of 0.5 μM mitoBHT in the presence of mitochondria. Addition of 4 mM succinate induced membrane potential and caused a decrease in external mitoBHT concentration. External mitoBHT concentration was largely restored when the membrane potential was dissipated with 0.3 μM FCCP. **(B)** Representative traces of oxygen consumption in the presence of mitoBHT. DMSO, different concentrations of mitoBHT, or 0.3 μM FCCP were present where indicated. **(C)** Double logarithmic plots of uncoupling in rat liver mitochondria. Titration of respiration with mitoBHT (open circles), mitoDNP (open triangles) or TPMP (squares). Closed circles: rate at each mitoBHT concentration after subsequent addition of 0.3 μM FCCP. Data are means ± SEM of four independent experiments. Lines were fitted by regression. **(D)** Effect of mitoBHT on the respiration of rat liver mitochondria in the presence of CAT. Double logarithmic plots of uncoupling in the presence of 2 nmol CAT/mg protein (added before succinate). Open circles: titration of respiration with mitoBHT. Closed circles: rate at

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each mitoBHT concentration after subsequent addition of 0.3 μM FCCP. Data are means ± SEM of four independent experiments. The line was fitted by regression. **(E)** CAT-sensitive stimulation of mitochondrial respiration rate by mitoBHT in linear coordinates. CAT-sensitive stimulation of respiration rate (open circles, shaded profile) was estimated as total uncoupling (filled circles; data from (C) where CAT was absent) minus CAT-insensitive respiration (open triangles; data from (D) where CAT was present). Lines were fitted as in Fig. 5E-H. **(F)** Kinetic response of proton leak to membrane potential of rat liver mitochondria incubated with 2 nmol CAT/mg protein, in the presence of DMSO and different concentrations of mitoBHT. Data are means ± SEM of four or five independent experiments. **(G)** Potential-independent uncoupling of rat liver mitochondria by mitoBHT. Data taken from (F) at the highest common membrane potential of 152 mV (solid line; slope = 0.69 ± 0.10; error bars for interpolated points are the weighted means of the error bars on flanking experimental points), or at the highest potential for each uncoupler concentration (dashed line; slope = 0.39 ± 0.04; points and error bars as in (F) but omitted here for clarity). The slopes are significantly different. **(H)** Effect of mitoBHT on the respiration of rat thymocytes, presented as a double logarithmic plot. Points are means ± SEM of three independent experiments. The slope of the regression line is 0.13 ± 0.05.

Figure 8. Effect of mitoQ and TPP compounds on the respiration of rat liver mitochondria.
(A-B) Double logarithmic plots of uncoupling in the presence (squares) and absence (circles) of 2 nmol CAT/mg protein (added before succinate). Open symbols: titration of respiration with (A) mitoQ_{10}. or (B) decylTPP. In the presence of CAT, concentrations of mitoQ_{10} or decylTPP below 10^{-7}M gave less than 1% increase in respiration and are off-scale. Closed symbols: rate at each mitoQ_{10} or decylTPP concentration after subsequent addition of 0.3 μM FCCP. Data are means ± SEM of three to four independent experiments. Lines (no CAT: dashed line; with CAT: dotted line) were fitted to the points shown by regression. **(C-D)** Comparison of uncoupling by a series of (C) mitoQ compounds and (D) TPP cations with different numbers of carbon atoms (from 3 to 10) in the alkyl chain (grey bars). The concentration used for this comparison was 10^{-9} M. At this concentration, CAT inhibited any uncoupling effect by these compounds (black bars). Data with mitoBHT (Fig. 7C, D) and methylTPP (TPMP; Fig. 7C) is included for comparison.

Figure 9. Hypothesis for the observed wide dynamic range of uncoupling by BHT and other compounds, illustrated as double logarithmic and linear plots.
Two main effects are responsible: (i) saturable, medium-capacity, high-affinity, CAT-sensitive uncoupling through the adenine nucleotide translocase at low BHT concentrations (modelled as a rectangular hyperbola with arbitrary K_m and V_{max}), and (ii) non-saturable, high-capacity, low-affinity, CAT-insensitive uncoupling through other pathways (such as conventional uncoupler cycling) at high BHT concentrations (modelled as a linear relationship). The wide dynamic range (i + ii) is the result of

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an overlap of these two effects. The right-hand panel shows these effects in linear co-ordinates; the left hand panel shows the same values plotted in log-log co-ordinates, illustrating how they can combine to give the shallow pseudo-linear overall relationship observed in the other Figs.

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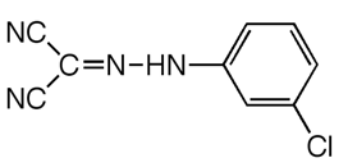
Figure 1



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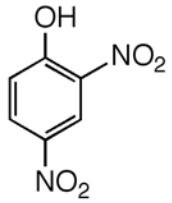

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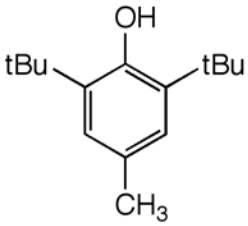

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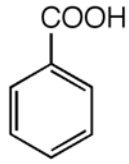
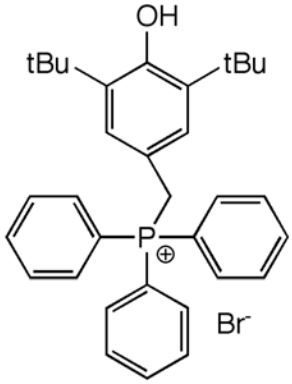
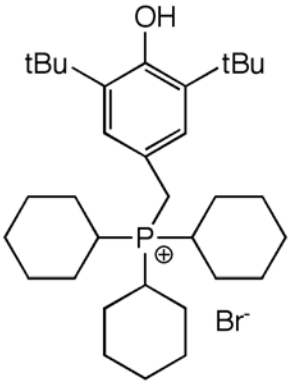


Benzoic acid



MitoBHT



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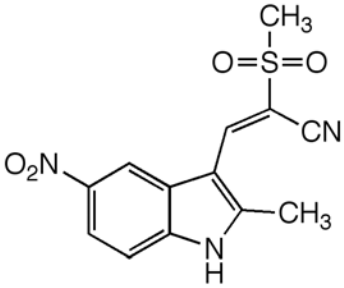

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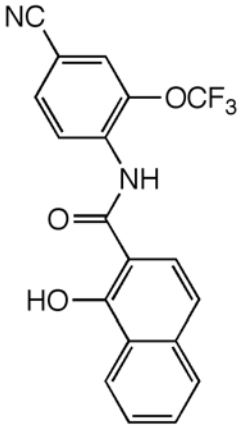

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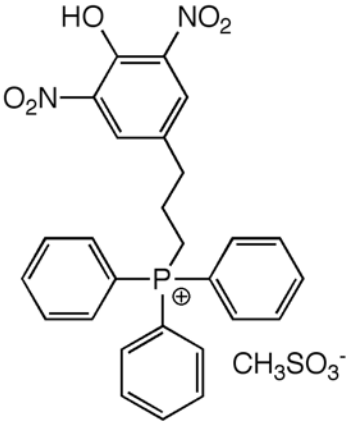

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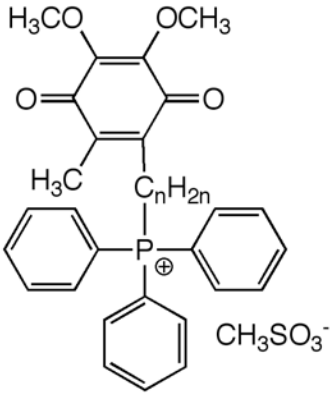

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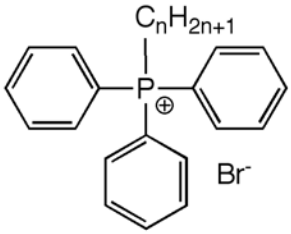

TPP series

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Figure 2

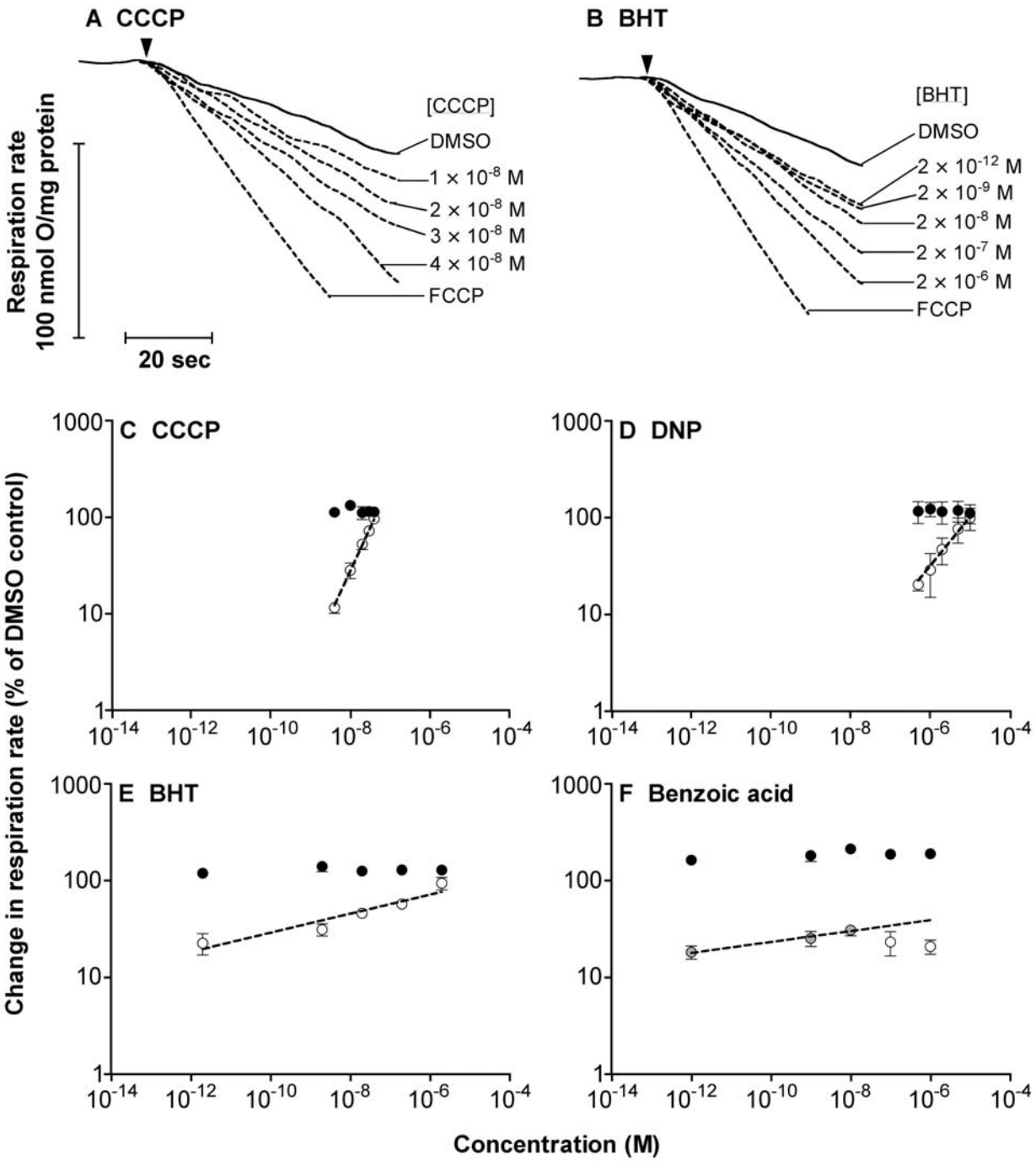

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Figure 3

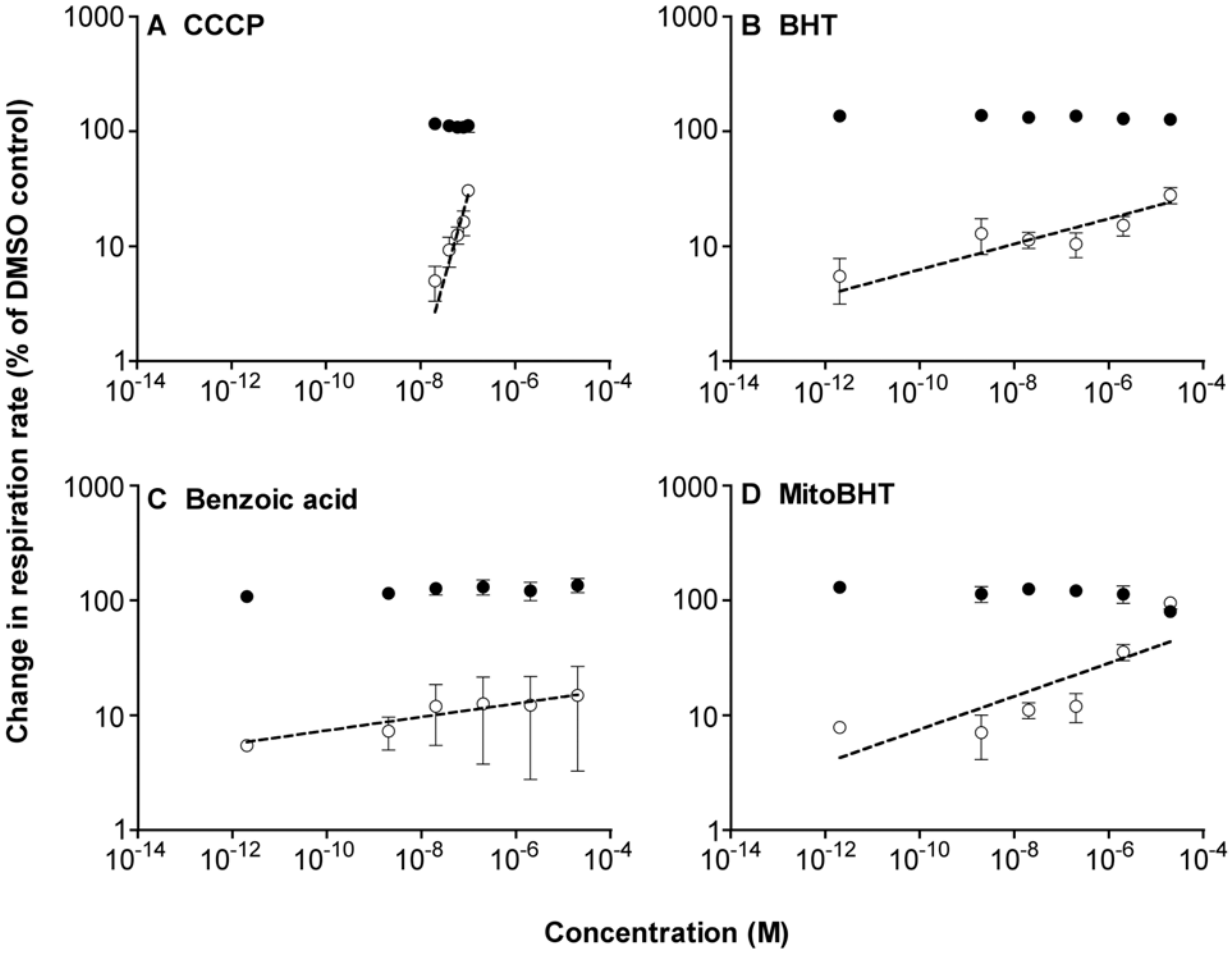

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Figure 4

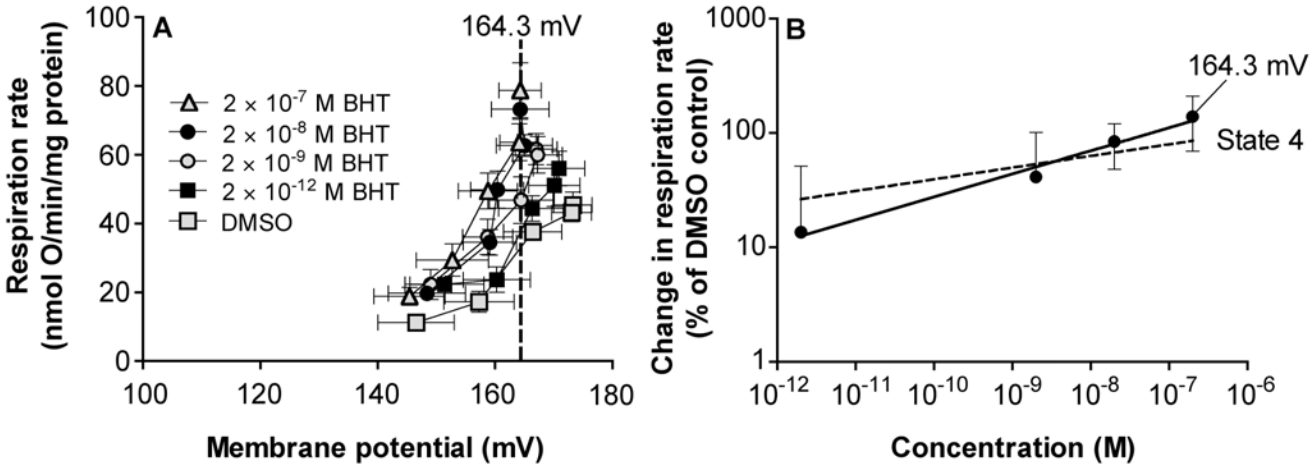

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Figure 5

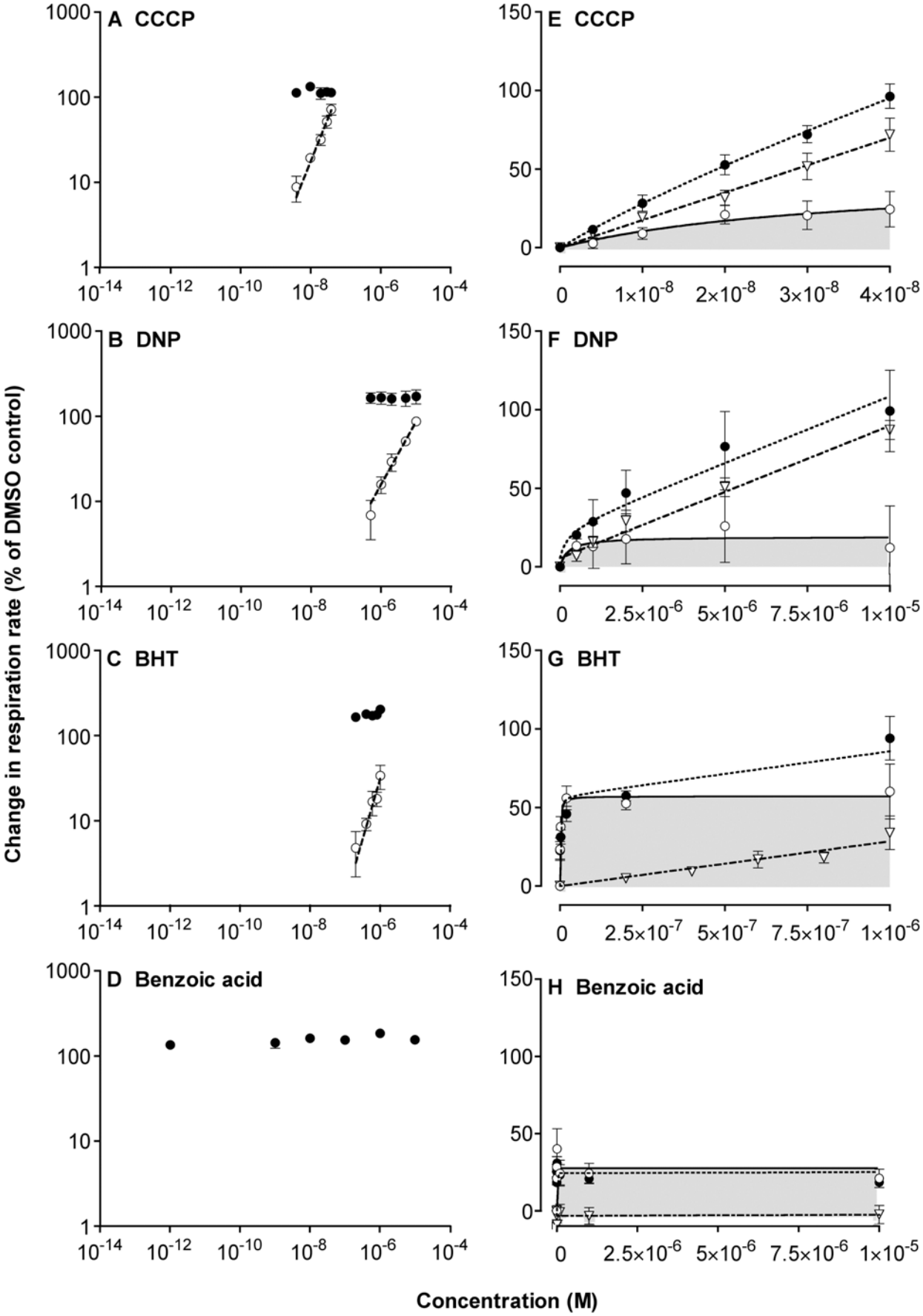

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Figure 6

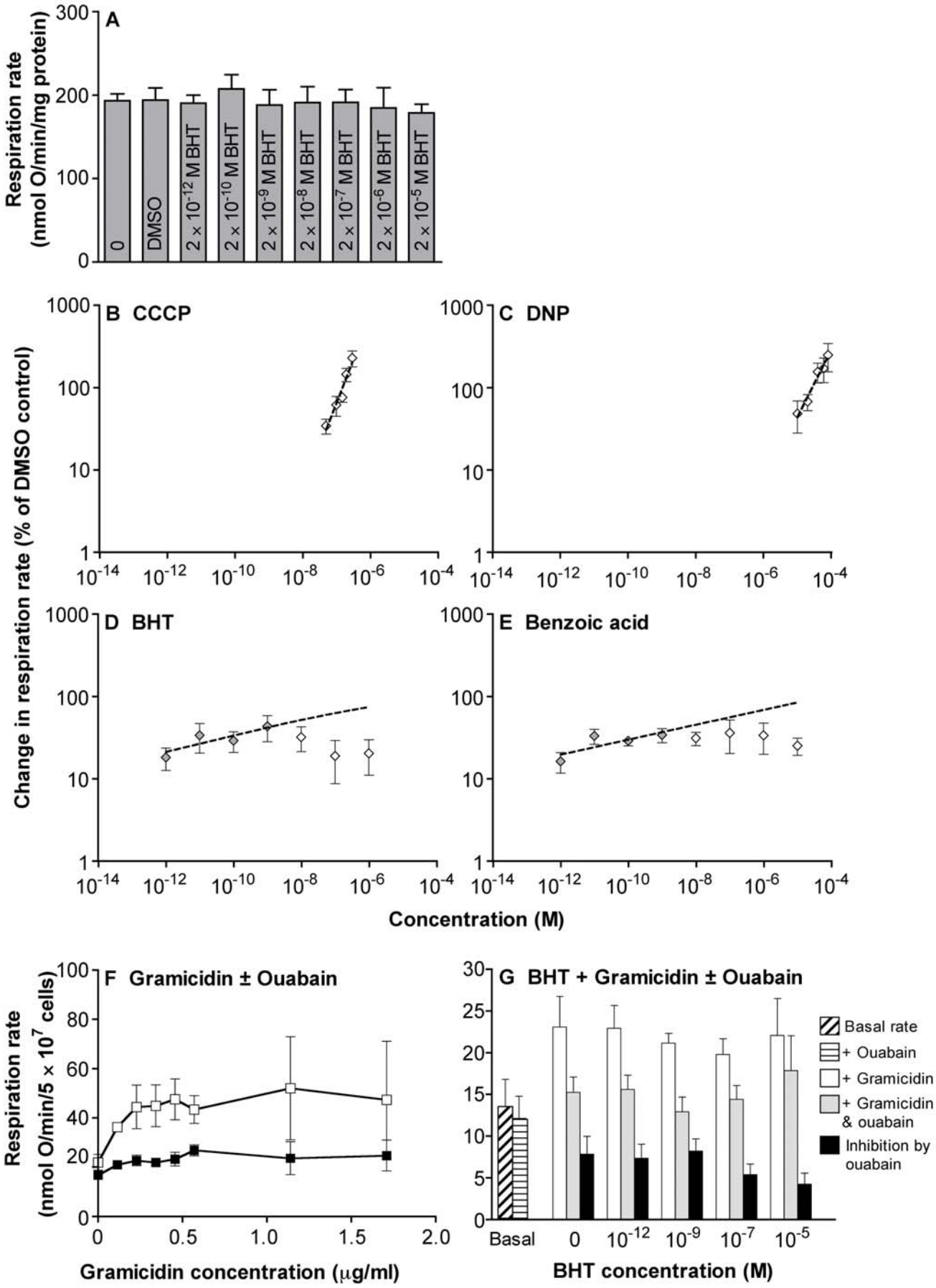

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Figure 7

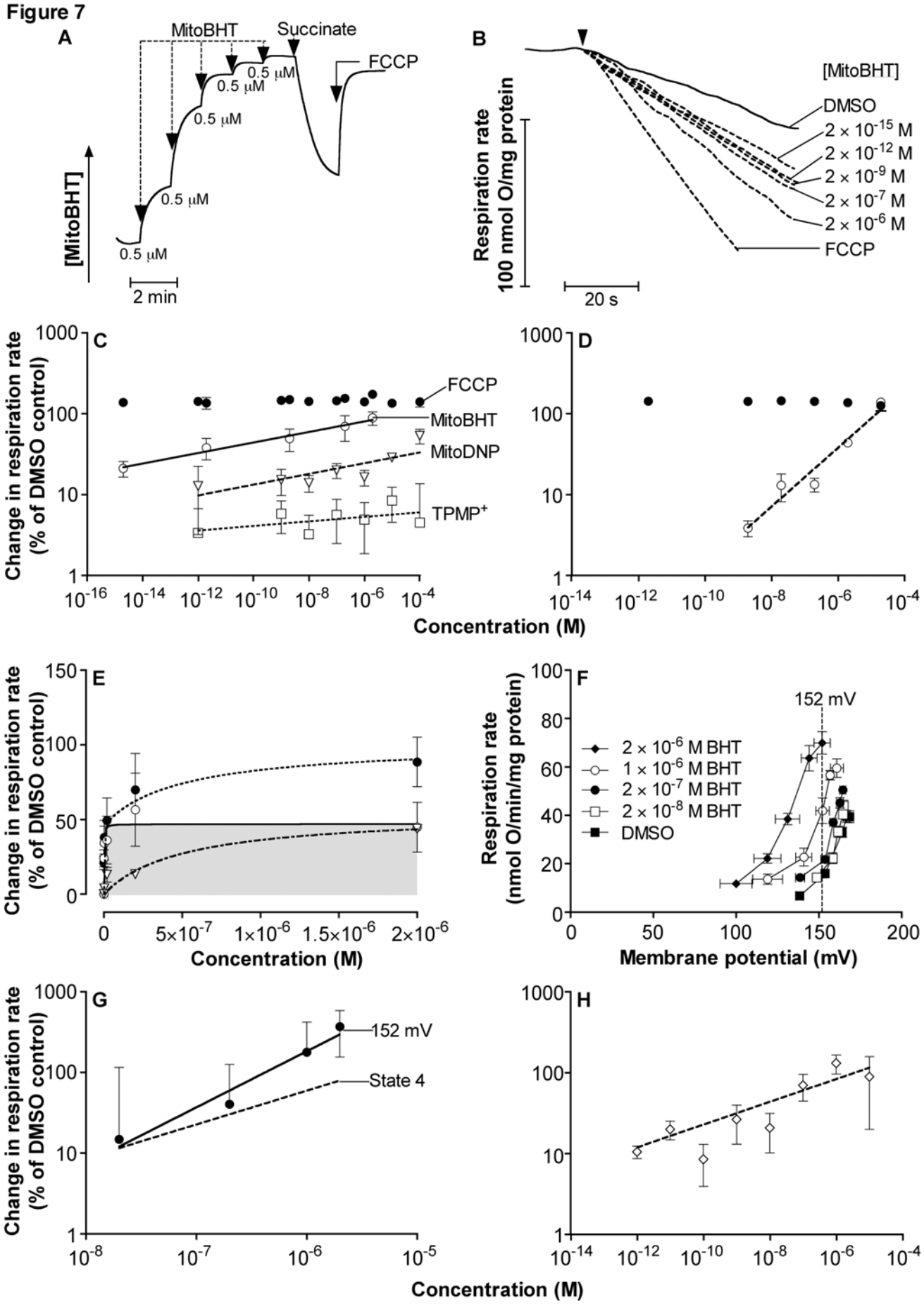

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Figure 8

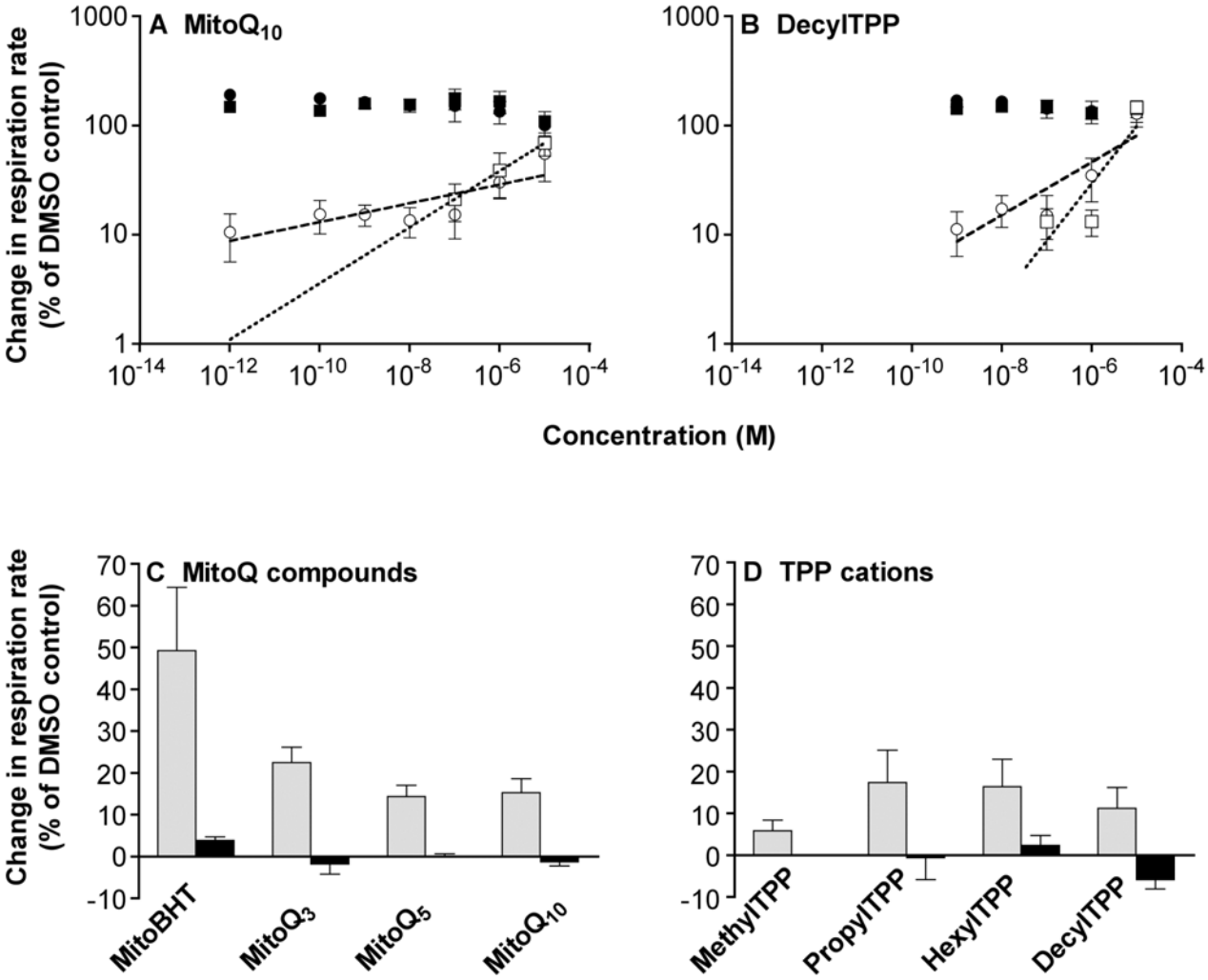

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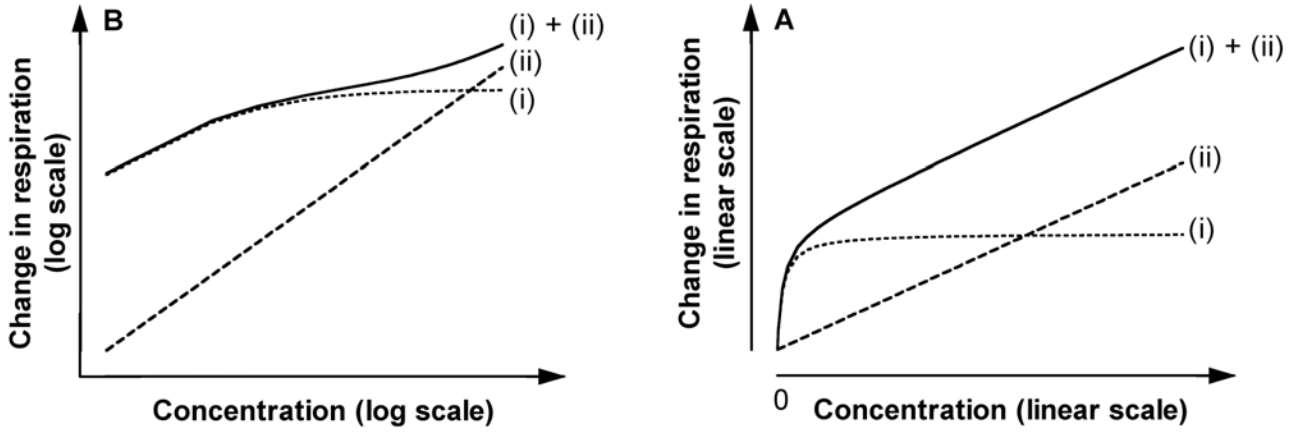
Figure 9



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