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THE ANTIANGINAL AGENT RANOLAZINE IS A WEAK INHIBITOR OF THE RESPIRATORY COMPLEX I, BUT WITH GREATER POTENCY IN BROKEN OR UNCOUPLED THAN IN COUPLED MITOCHONDRIA

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Abstract—Ranolazine (RS-43285) has shown antianginal effects in clinical trials and cardiac anti-ischaemic activity in several *in vivo* and *in vitro* animal models, but without affecting haemodynamics. Its mechanism is thought to mainly involve a switch in substrate utilisation from fatty acids to glucose to, thus, improve efficiency of O_2 use; however, its precise molecular target(s) are unknown. In studies to investigate its action further, using isolated rat heart mitochondria, ranolazine was found to weakly inhibit (pIC_{50} values > 300 μM) respiration by coupled mitochondria provided with NAD^+-linked substrates but not with succinate. With broken mitochondrial membranes or submitochondrial particles, ranolazine inhibited NADH but not succinate oxidation and with pIC_{50} values in the lower range of 3–50 μM. Studies with different electron acceptors and respiratory inhibitors indicated that it inhibits respiratory Complex I at a site between ferricyanide and menadione and ubiquinone-1 reduction (*i.e.* at a similar locus to rotenone). However, unlike rotenone, ranolazine was an uncompetitive inhibitor with respect to ubiquinone-1. Ranolazine inhibition of Complex I was reversible and occurred also with mitochondria from pig, guinea pig, and human heart, and rat liver. Further studies using rat heart mitochondria in different energisation states (*i.e.* broken, uncoupled, or coupled) showed a 50–100-fold shift to greater potency of ranolazine in the broken compared to the coupled; with the uncoupled it was about 2-fold less potent than the broken. These shifts in potency were not found with rotenone or amytal. Studies with radiolabelled ranolazine showed that it bound to mitochondrial membranes with greater affinity in the broken compared to the coupled or uncoupled conditions. Rotenone displaced radiolabelled ranolazine from its binding site. This property of ranolazine may play some role in its anti-ischaemic activity.

Key words: ranolazine; respiratory Complex I; mitochondria; NADH dehydrogenase; amytal; rotenone

Ranolazine (RS-43285; (±)-N-(2,6-dimethylphenyl)-4[2-hydroxy-3(2-methoxyphenoxy)-propyl]-1-piperazine acetamide), a novel investigational antianginal agent, has shown efficacy in clinical trials [1–4], and cardiac anti-ischaemic activity in a number of *in vivo* [5–8] and *in vitro* [9–12] animal models, but without affecting haemodynamics or baseline contractile parameters. Its mechanism of action is thought to principally involve causing a switch in myocardial substrate oxidation from fatty acid to glucose, and evidence to support this has been obtained [13–15]. Such a switch may improve the efficiency of O_2 utilisation by the heart, which would have special significance under conditions of O_2 limitation, and also has a number of other potential advantages for protection of the ischaemic heart [15–17]. It is not known in molecular detail how this metabolic modulation is achieved, although increases in the amount of active nonphosphorylated pyruvate dehydrogenase, the key rate-controlling enzyme in carbohydrate oxidation [18], have been found in hearts exposed to ranolazine [11, 19].

Pyruvate dehydrogenase and other key enzymes in metabolic substrate oxidation are located within mitochondria in mammalian tissues (see [20]) and we, therefore, initiated a series of experiments with ranolazine using isolated rat heart mitochondria. During the course of this work, we found that ranolazine had some effects on the respiration of NAD^+-linked substrates. Free radical formation by respiratory complexes is thought to be associated with the mitochondrial damage that occurs in cardiac ischaemia/reperfusion, which is thought to be due to peroxidation of lipid components of the respiratory chain Complexes themselves [21–23]. It has also been reported that blocking mitochondrial respiration with amytal, a weak respiratory Complex I inhibitor, significantly reduces mitochondrial lipid peroxidation in rabbit hearts reperfused after 30 min global ischaemia [24]. We have, therefore, investigated the effects of ranolazine on the mitochondrial respiratory chain in some detail.

MATERIALS AND METHODS

Chemicals

Ranolazine was synthesized and provided by Syntex Research (address as above); [^{14}C]- and [^{3}H]-ranolazine were synthesised and provided by Dr. Howard Parnes, Syntex Discovery Research, 3401 Hillview Ave., Palo

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§ Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Alto, CA, U.S.A. Ubiquinone-1 was a generous gift of Eisai Co. Ltd., Tokyo, Japan. Nagarse (Protease Type XXVII), menadione, and capsaicin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and 1, 2-naphthoquinone and rotenone were from Janssen Chimica, Beerse, Belgium. All other chemicals were of the highest grade available from standard suppliers.

Mitochondrial preparations

Experiments were performed in both laboratories and, in some cases, slightly different procedures were used as noted below; however, essentially similar results were obtained when the same type of experiment was performed in both laboratories. Thus, respectively, in Brussels anaesthetised (nembutal) fed male Wistar rats of ~200 g weight were used and, in Edinburgh, cervically dislocated and decapitated male Sprague-Dawley rats of ~300 g weight were used. Standard differential centrifugation techniques were used. Respectively, liver mitochondria were prepared in 0.3 M mannitol, 10 mM HEPES, 1 mM EGTA (pH 7.4) as described [25], or in 250 mM sucrose, 20 mM Tris, 2 mM EGTA, 1% (w/v) (largely defatted) bovine serum albumin (BSA), pH 7.4 ("STEA" buffer), and with a Percoll purification step and final suspension in buffer minus albumin ("STE" buffer) as described [26]. Respectively, heart mitochondria were prepared in 220 mM mannitol, 70 mM sucrose, 5 mM MOPS, 1 mM EGTA (pH 7.4), and employing a 5 min nagarse (0.5 mg/ml) digestion as fully described [27], or using a brief Polytron homogenisation in STEA buffer and final suspension in STE buffer as described [26]; guinea pig (female, Dunkin-Hartley, approx. 300 g) heart mitochondria were also prepared by the latter method. Pig and human heart mitochondria samples provided by Prof. Sherratt (see Acknowledgements) were prepared as described [28] for muscle. Final suspensions were in the range of 15–40 mg protein/mL. Protein was assayed by either the Lowry method or using the Pierce (Rockford, IL, U.S.A.) kit, respectively.

Broken mitochondrial membranes were prepared by freeze-thawing final suspension samples (in suspension buffer that had been stored frozen) 3 times using liquid nitrogen. Submitochondrial particles were prepared (in Brussels) essentially as described [29].

Mitochondrial incubations

Mitochondrial respiration (0.5–2 mg protein) was measured at 30°C using Clark-type oxygen electrodes in 1–3 mL buffer containing either 120 mM KCl, 2.5 mM KH_2PO_4, 10 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, 2 mg/mL defatted BSA (pH 7.2), or 125 mM KCl, 5 mM KH_2PO_4, 20 mM Tris, 1 mM EGTA (pH 7.3), in Brussels and Edinburgh, respectively. Substrates used included 5 mM glutamate plus 5 mM malate, 10 mM 2-oxoglutarate plus 0.5 mM malate, or 5 mM succinate (±2 μg/mL rotenone) as substrates. "State 3" respiration was initiated with either 0.25 mM or 2 mM ADP. Oxygen concentration was taken to be 0.45 μg atoms/mL at 30°C.

With broken mitochondrial membranes, respiration was measured, as above, after the addition of either 0.25 or 1 mM NADH, respectively, or 5 mM succinate (±2 μg/mL rotenone). Respiratory Complexes were assayed using these preparations or using submitochondrial particles at 30°C and exactly as described [29]. Briefly, for membranes, NADH-ubiquinone oxidoreductase (Complex I) was assayed as rotenone-sensitive NADH oxidation at 340 nm in a thermostatted stirred cuvette; membranes (10–25 μg protein) and 100 μM ubiquinone-1 (UQ-1) were preincubated for 3 min at 30°C in 1 mL of 35 mM potassium phosphate (pH 7.2), 5 mM $MgCl_2$, 2 mg defatted BSA, 2 μg antimycin A, and 2 mM KCN; drugs were added immediately after membranes. The initial linear rate was followed for 1 min after the addition of 0.13 mM NADH, and then after adding 5 μg of rotenone. An extinction coefficient of 6.81 mM/cm was used for NADH to correct for change in absorbance of UQ-1 [29]. Similarly, NADH oxidation was also assayed with 100 μM menadione and 100 μM 1,2-naphthoquinone as electron acceptors in the same buffer in the presence of rotenone (5 μg/mL), in which case E values of 6.22 mM/cm or 5.0 mM/cm were used, respectively. NADH-ferricyanide oxidoreductase activity was assayed at 420 nm (E = 1.05 mM/cm) in 35 mM potassium phosphate (pH 7.2), 5 mM $MgCl_2$, 2 mM KCN, 0.5 mM $K_3Fe(CN)_6$ with 0.5 mM NADH (a ratio of 2 molecules of ferricyanide reduced per molecule of NADH was assumed to allow expression of activities in terms of μmol of NADH). NADH- and succinate-cytochrome *c* oxidoreductase were measured at 550.5 nm (E = 18.5 mM/cm) in 35 mM potassium phosphate (pH 7.2) 5 mM $MgCl_2$, 2 mM KCN, 50 μM cytochrome *c*, with 0.13 mM NADH and 20 mM succinate being added as respective substrates. Essentially similar conditions were used for submitochondrial particles, but with some minor modifications (see legend to Fig. 2).

Radioligand binding

Incubations for the binding of [^{14}C]-ranolazine to mitochondria were designed to match as closely as possible the respiratory experiments and, hence, used essentially the same conditions and buffers as described above (Edinburgh), except that about 3–5 times the amount of mitochondrial protein/mL was present to increase amounts of bound ligand and enable easier measurements and lessen experimental error. Thus, either 10 mM 2-oxoglutarate plus 0.1 mM malate alone, or plus 1 μM FCCP,§ or 1 mM NADH were present for incubations under coupled, uncoupled, or broken mitochondrial conditions, respectively. Incubations at different ranolazine concentrations and containing suitable amounts of [^{14}C]-ranolazine were carried out in 1 mL volume at 30°C for 15 min in Eppendorf microfuge tubes. The tubes were then centrifuged in a microfuge for 1 min, the supernatant removed by syringe, and the sides of tubes dried using cotton buds. Formic acid (200 μL) was added to solubilise each pellet and the radioactivity in each was determined by liquid scintillation counting.

For experiments with [^{3}H]-ranolazine, aliquots of mitochondria (~0.5 mg protein) were incubated in 1 mL of 0.25 M sucrose, 0.5 mM HEPES (pH 7.4), and with ligand at 10–40 nM containing suitable amounts of [^{3}H]-ranolazine. In competition experiments, unlabelled ranolazine or rotenone were present over the range 10^{-10} to 10^{-4} M. Incubations were carried out at 25°C for 45 min and separation of bound from free ligand was carried out using a Brandel M24 cell harvester over Whatman GF/B filter pads that had been previously soaked in 0.5% polyethyleneamine; radioactivity bound to filters was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 1 mM unlabelled ranolazine.

Data analysis

Where appropriate, results are given as means ± SEMs or as IC_{50} values ± SD for the numbers of separate mitochondrial preparations given in parentheses. A suitable range of concentrations was used in IC_{50} determinations; $pIC_{50} = -\log$ of the IC_{50}. Statistical comparisons have been made with an unpaired student's *t*-test.

RESULTS

Ranolazine is thought to influence metabolic substrate utilisation (see Introduction). Because the endpoint metabolism of the principal substrates is carried out by mitochondria, and their metabolic fate can be controlled by enzymes within mitochondria, we began studies on the effects of ranolazine on isolated mitochondrial function.

Effects of ranolazine on respiratory substrate oxidation

In coupled rat heart mitochondria, the State 3 (plus ADP) oxidation rate of 2-oxoglutarate plus malate (*i.e.* NAD^+-linked substrates) was found to be inhibited in a concentration-dependent manner by high concentrations of ranolazine (IC_{50} > 300 μM) (see Fig. 1). These high concentrations may indicate a nonspecific action of the drug (but see below). State 4 respiratory rates and ADP:O ratios were unaffected by ranolazine (not shown). These types of experiments were carried out with two protocols; one where ranolazine was added 1–2 min before respiratory substrate, and one where it was added 1–2 min after the (control) State 3 rate with respiratory substrate had been established. In the former instance, the inhibited rates with NAD^+-linked substrate were linear throughout; however, in the latter the rates curved down over 1–2 min before the new inhibited rates became linear. Similar resultant linear rates were obtained with each protocol, but the results with the latter suggest that the drug needs some time to permeate to its site of action in intact mitochondria (see below and Discussion later). Similar results (not shown) to those in Fig. 1 were also obtained with other NAD^+-linked substrates, such as glutamate plus malate, pyruvate, or β-hydroxybutyrate. In contrast, succinate oxidation was unaffected at all concentrations of drug tested (up to 1.3 mM) (Fig. 1). As several different NAD^+-linked substrates were affected, this suggested that the ranolazine site of inhibition was the respiratory Complex I rather than any of the dehydrogenases or transport processes and, therefore, studies were carried out using broken mitochondrial membranes as a simpler system containing all the respiratory Complexes.

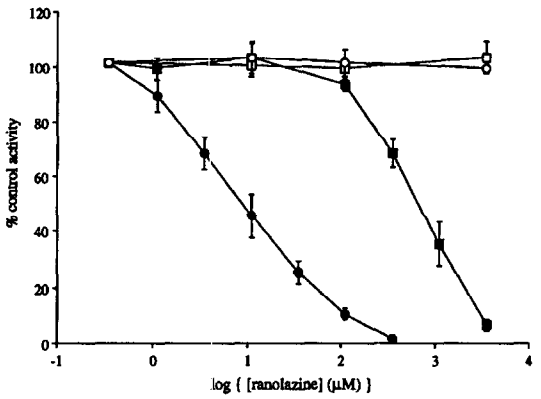


Fig. 1. The effects of ranolazine on State 3 respiration in coupled rat heart mitochondria with different substrates, and on NADH and succinate oxidation by disrupted rat heart mitochondrial membranes. Incubations were carried out in Edinburgh as described in the Methods section for coupled mitochondria in the presence of 2 mM ADP and either 10 mM 2-oxoglutarate plus 0.5 mM malate (filled squares) or 5 mM succinate (open squares), and for broken membranes in the presence of either 1 mM NADH (filled circles) or 5 mM succinate (open circles). Values and error bars shown are means ± SEM for measurements made on 3 different mitochondrial preparations; derived $K_{0.5}$ values (μM) of 9.8 ± 1.6 and 575 ± 84 were obtained for the broken membrane and coupled mitochondria NADH-linked respiration, respectively, in this set of experiments. Similar results for succinate respiration were obtained in the additional presence of rotenone, and essentially similar results to those shown were obtained in the Brussels laboratory and in other experiments in Edinburgh; from all these experiments $K_{0.5}$ values in the approximate ranges of 3–50 μM and 300–1800 μM were obtained for the broken membranes and coupled mitochondria, respectively.

Thus, as predicted, in broken mitochondrial membranes incubated with NADH as substrate, ranolazine inhibited respiration. However, this was with apparently greater potency than in the experiments with intact coupled mitochondria, and with IC_{50} values in the 3–50 μM range (see Fig. 1). This apparent change in potency of ranolazine is explored in greater detail later (see below). In contrast to the case with intact mitochondria, the inhibition of NADH oxidation by ranolazine was observed immediately on addition of the drug with the broken membrane preparations. Also, in experiments where the membranes were exposed to drug at inhibitory concentrations and were then re-isolated by centrifugation and suspension into media in the absence of drug, evidence was obtained that the effects were readily reversible (not shown). Again, succinate respiration, as predicted, was unaffected in the broken mitochondrial membranes (Fig. 1). These experiments, thus, confirm that ranolazine is an inhibitor of the respiratory Complex I; this was then explored in more detail (next heading).

It is worth noting that essentially similar findings to those shown in Fig. 1 for rat heart mitochondria were also found for rat liver mitochondria (not shown). However, as ranolazine is an antianginal agent, all subsequent studies described below were on heart mitochondria. Although it is likely that similar findings to those below would have been found with liver mitochondria and, given the ubiquity of the respiratory Complexes, with mitochondria from other tissues as well. It was also established, in studies on NADH and succinate oxidation using mitochondrial membranes, that ranolazine inhibited Complex I activity of mitochondria prepared from hearts of guinea pigs, pigs, and humans (not shown), and with similar potency to that shown by the rat heart Complex (pIC_{50} values in the 3–50 μM range).

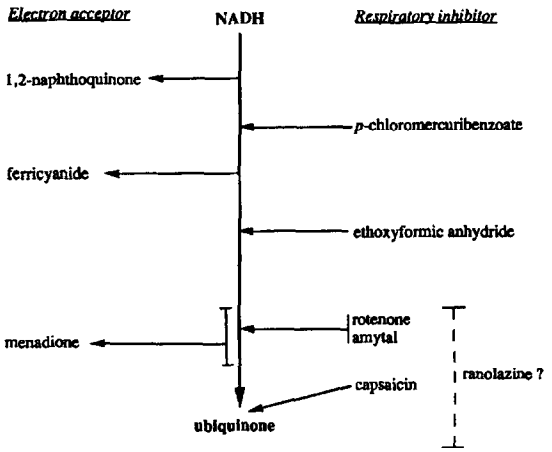
Studies with artificial electron acceptors and respiratory inhibitors

Experiments were carried out using submitochondrial particles incubated with such agents to determine the site of interaction of ranolazine within the respiratory Complex I (*i.e.* the NADH-ubiquinone span of the chain). It should be noted that essentially similar findings were also obtained using broken mitochondrial membranes;

however, as might be expected (see next heading and Discussion later) ranolazine was typically slightly more potent in its effects using the completely de-energised broken membranes (not shown).

Thus, with reference to Scheme 1, which is derived from data described [30–33], Fig. 2 indicates that the activities obtained using ferricyanide or 1,2-naphthoquinone were unaffected by ranolazine up to 1 mM, whereas the activities with menadione or UQ-1 were inhibited in a similar manner to that when oxygen (*e.g.* Fig. 1) or cytochrome *c* (not shown) were the terminal acceptors. This pattern of inhibition is similar to that expressed (but at higher potency) by rotenone (not shown). The remaining rotenone-insensitive rates represent NADH-cytochrome b_5 activity associated with the outer mitochondrial membrane and have been subtracted from rates shown; this rotenone-insensitive activity [29] was typically ~10–30% (dependent on the terminal acceptor) of the total activity in such preparations and was not affected at all by ranolazine (not shown).

NADH-ubiquinone oxidoreductase can be inhibited by several compounds, including rotenone, amytal, and capsaicin. Capsaicin (*trans*-8-methyl-vanillyl-6-nonenamide) has also been shown to inhibit NADH-ubiquinone oxidoreductase at a site subsequent to the menadione site [34] and, moreover, it has been shown to competitively inhibit ubiquinone binding [30]. We, therefore, tested the effect of ranolazine on NADH-ubiquinone oxidoreductase activity at different [UQ-1] and compared it with capsaicin (Fig. 3). The double-reciprocal plot clearly revealed competition between 50 μM capsaicin and the varied [UQ-1], its apparent K_m being increased from 44 to 105 μM but with no change in V_{max}. In contrast, the pattern of ranolazine inhibition when tested at 5 μM was that of an uncompetitive inhibitor with respect to UQ-1, with the K_m decreasing to 12.4 μM with a 70% decrease in V_{max}. Thus, the site of ranolazine interaction with Complex I appears to lie between that of menadione and ubiquinone (Scheme 1). Although the nature of its inhibition (Fig. 3) suggests some interaction between ranolazine and ubiquinone, it is not due to competition for the ubiquinone binding site, unlike capsaicin. It should be noted that, in a similar experiment, rotenone had no affect at all on the ubiquinone K_m and only decreased the V_{max} [35]; thus, although acting in a similar locus to rotenone, the mechanism of respiratory inhibition by ranolazine is different in some ways to that of rotenone.



Scheme 1. The sequence of reactions of electron acceptors and respiratory inhibitors within Complex I in the transfer of electrons from NADH through to ubiquinone. These positions are derived from the data given before [30–33] and the present experiments with ranolazine.

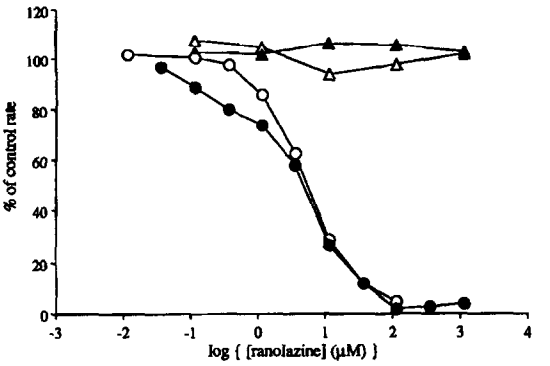


Fig. 2. The effects of ranolazine on NADH oxidation by rat heart submitochondrial particles incubated (see Methods) with different artificial electron acceptors. All values shown are from duplicate assays from a typical experiment that was repeated with at least 3 different mitochondrial preparations. The respective control values in this experiment (as μmol NADH/min/mg protein) were 3.4 with 500 μM ferricyanide (open triangles), 0.29 with 500 μM menadione (filled circles), 3.68 with 100 μM 1,2-naphthoquinone (filled triangles), and 1.87 with 100 μM ubiquinone-1 (open circles).

The differential potency of ranolazine with variation in mitochondrial energisation state and its comparison to other Complex I inhibitors

We wished to explore further the apparent shift in potency of ranolazine's action on Complex I as the mitochondrial energisation state was changed (Fig. 1), and to see whether this was a relatively unique property of ranolazine or a more general phenomenon for Complex I inhibitors. In particular, we chose to compare ranolazine with rotenone and amytal, which similarly inhibit Complex I after the menadione site, with one (rotenone) being a very potent inhibitor and amytal's potency in a

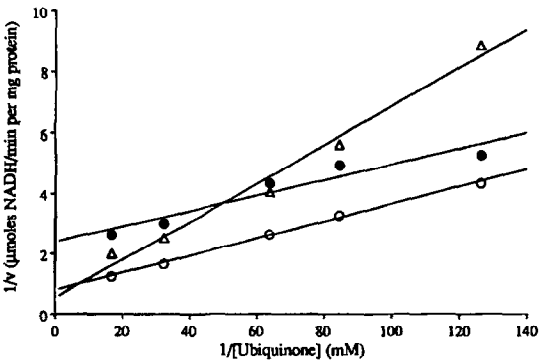


Fig. 3. Double-reciprocal plot of rat heart submitochondrial particle NADH-ubiquinone reductase activity as a function of ubiquinone-1 concentration under control conditions (see Methods, open circles) and in the presence of either 5 μM ranolazine (closed circles) or 50 μM capsaicin (triangles). A typical experiment, which was repeated with at least 3 different mitochondrial preparations, is shown.

Table 1. Comparative potencies of ranolazine, amytal, and rotenone in inhibiting respiratory Complex I under different conditions of energisation of the mitochondrial inner membrane

Inhibitor	IC_{50} value for inhibition of Complex I in mitochondria that are:		
	Coupled	Uncoupled	Broken
Amytal (μM)	637 ± 52	892 ± 120	539 ± 54
Rotenone (nM)	38.8 ± 3.2	49.1 ± 4.1	33.5 ± 2.1
Ranolazine (μM)*	1584 ± 340	754 ± 154	22.7 ± 3.8

See Methods section for details of incubation conditions for each mitochondrial energisation state (these experiments were performed in Edinburgh). Values are means ± SEM for three independent estimations of $K_{0.5}$ values derived over suitable concentrations of inhibitors on different preparations of mitochondria.

* The values obtained with ranolazine are all significantly different from one another ($P \leq 0.05$); the values for the other inhibitors (comparing the different energisation states) were not statistically different from one another.

similar range to that for ranolazine. Table 1 shows that inhibition of Complex I by amytal (or rotenone) does not vary substantially as mitochondrial energisation state is changed from them being fully coupled to fully disrupted membranes. In contrast, whereas ranolazine shows about 2.5-fold lesser potency than amytal in the coupled mitochondria, it is about 25-fold more potent than amytal in the broken mitochondria (Table 1). With mitochondria uncoupled with FCCP the potency of ranolazine and amytal is similar (Table 1).

Trimetazidine, another antianginal agent that is thought to be a metabolic modulator [36] and which has recently been shown to inhibit mitochondrial respiration with fatty acid as substrate [37], was also tested. However, it did not inhibit Complex I very potently when assayed under the present conditions ($IC_{50} > 1$ mM) and its potency did not alter between the different energisation states of mitochondria (not shown). All this strongly suggests that ranolazine may have a unique action in this respect.

Studies using radiolabelled ranolazine

The experiments shown in Table 2 were designed to match as closely as possible the respiratory experiments of Table 1 to see whether the amount of ranolazine bound to mitochondrial membranes under different energisation states would change in a parallel manner to the observed shifts in potency (Table 1). [^{14}C]-ranolazine was available at the time of these experiments and Table 2 shows that the amount of radiolabel associated with mitochondria incubated at two different ranolazine concentrations did, indeed, seem to be higher (though only about 3–5-fold) when broken mitochondrial membranes were used in comparison to fully coupled mitochondria; however, the uncoupled mitochondria showed a similar degree of binding as the coupled (Table 2). This data, therefore, fits to some extent with that in Table 1 (where increased Complex I inhibitory potency is seen with the broken membranes), and suggests that more ranolazine may bind to the broken membranes and that this may be part of the reason underlying the differential potencies observed. However, the results comparing the uncoupled and coupled suggest also that the degree of energisation *per se* is also important, as increased potency appears to be evident in the uncoupled for a similar degree of binding.

Some experiments were also carried out with [^{3}H]-ranolazine when it was available. The conditions used (see Methods) were chosen so that classical competition binding assays could be performed, but would almost certainly mean that mitochondria would be in a de-energised state (no substrates were present). This is reflected in the fact that the apparent affinity (pIC_{50}) for unlabelled ranolazine in these experiments was calculated [38] to be 5.76 ± 0.15. These experiments also revealed that ranolazine rapidly associated ($t_{1/2}$ approx. 2.5 min with 10 nM labelled ranolazine (at 25°C)) and dissociated ($t_{1/2}$ approx. 3 min with 10 μM unlabelled displacing 10 nM labelled) with the mitochondria, and that the two stereoisomers were equipotent in this competitive effect against radiolabelled racemate (not shown). In the association experiments, binding reached equilibrium within 10 min and was stable for a further 40 min. Of great interest was that rotenone was also found to displace the radiolabelled ranolazine with a pIC_{50} of 7.59 ± 0.09, which is close to its respiratory inhibition potency (see above). This also suggests that ranolazine may interact with the rotenone site.

Table 2. The binding of ranolazine to mitochondrial preparations under different states of energisation of the inner membrane

Ranolazine concentration (μM)	Ranolazine bound (nmol/mg protein) to mitochondrial membranes under the different energisation states		
	Coupled	Uncoupled	Broken
100	0.96 ± 0.10 (7)	1.03 ± 0.28 (5)	5.25 ± 1.11 (7)*
1000	24.2 ± 6.1 (7)	22.6 ± 1.9 (5)	70.2 ± 10.7 (7)*

Values are means ± SEM for the number of values given in parentheses.

* $P \leq 0.01$ versus the appropriate coupled or uncoupled values. See Table 1 legend for further details.

DISCUSSION

We have shown that ranolazine inhibits mitochondrial respiration of NAD^+-linked substrates and that this is due to inhibition of electron transport at the level of Complex I. The exact sequence of reactions in this section of the respiratory chain has not been fully elucidated, but it is known to be sensitive to a variety of inhibitory compounds acting at different sites, notably capsaicin, mercurials, ethoxyformic anhydride, rotenone, and amytal [30–33] (Scheme 1). The inhibition by ranolazine was further shown to most closely resemble that of rotenone, amytal, and capsaicin, which inhibit between the sites of menadione and ubiquinone reduction. With the Complex I assays used, the half-maximal effect of ranolazine is around 10-fold higher and 10-fold lower than those of rotenone [39] and capsaicin [30], respectively. However, the double-reciprocal plots show that ranolazine, unlike capsaicin, is not a competitive inhibitor with respect to ubiquinone; they do indicate that, unlike rotenone, it may have some interaction with ubiquinone. However, the studies with [^{3}H]-ranolazine indicate that rotenone can displace ranolazine from mitochondrial membranes suggesting a proximity to the rotenone site (Scheme 1), and other features of its respiratory inhibition resemble those for rotenone (Fig. 2).

It is interesting to note that several piperidine derivatives, including the analgesic drug pethidine, are potent inhibitors of Complex I [39]. This suggests that structural similarities between piperidine compounds and ranolazine (a piperazine derivative) may explain its effect on Complex I. Thus, of even greater interest is the recent finding that both cinnarizine and flunarizine inhibit Complex I in rat liver, heart, and brain mitochondria; however, unlike ranolazine, these other two piperazine derivatives were additionally found to inhibit Complex II [29]. Also unlike ranolazine, these other two agents are known to be active in the central nervous systems (CNS) (ranolazine appears to have little or no access to the CNS according to unpublished observations of Syntex Research) and, thus, it has been proposed [29] that the respiratory inhibition effects of flunarizine and cinnarizine could be factors in the aggravation or induction of Parkinsonism associated with these compounds in elderly patients. That is, it is suggested [29] that they might act in a manner similar, but perhaps less selectively, to that of the Parkinsonism-inducing mitochondrial toxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine) whose toxic metabolite (MPP^+) is a Complex I inhibitor and is preferentially taken up into nigrostatial cells by the synaptic dopamine transport system [40, 41].

The differences in concentration-response observed between intact fully coupled mitochondria and broken mitochondrial membranes suggest that ranolazine might act at a site on the inner surface of the membrane (like NADH) not normally exposed to ranolazine in the intact organelles. However, as noted (see text for Fig. 1), with intact mitochondria the effect of ranolazine took some time to develop whereas with broken membranes it was instantaneous, suggesting that it can eventually permeate to its site of action. Also, the experiments with uncoupled mitochondria (where, as with the coupled mitochondria, the NADH is generated within the matrix from the respiratory substrate) would argue against the difference in potency being entirely due to access to its site in intact mitochondria unless, perhaps, if the permeabilisation of the membrane to H^+ also increases its permeability to ranolazine. Thus, the data overall suggest that, while access may play some role (perhaps explaining the difference between the uncoupled and broken preparations in potency (Table 1) and binding (Table 2), the more dominant factor may be the degree of energisation of the inner membrane which can influence whether or not ranolazine has greater or lesser access to its Complex I inhibitory site. Ranolazine has pK_a values of 2.8 and 7.2 and, thus, at around physiological pH approx. 50% of ranolazine will have a positive charge, but the amount of charged compound will be very sensitive to the pH of its environment. It is, thus, tempting to speculate either that (1) it is the combination of the membrane potential across the inner membrane and the charge on the compound that dictates its position within the membrane and its accessability to the inhibitory site; or (2) it is, perhaps, only the charged form of the compound that is inhibitory. However, in a series of experiments (not shown) carried out with broken membranes incubated at different pH values over the range 6.7 to 7.8, no substantial changes in potency of ranolazine were observed due to pH.

This change in potency of ranolazine dependent upon the energisation state of the mitochondria is certainly worthy of further consideration, especially as the evidence obtained in comparison to other Complex I inhibitors indicates that it may be somewhat unique in this respect (Table 1). This is emphasized by the fact that the piperazine compounds flunarizine and cinnarizine, mentioned above, also do not appear to exhibit this change in sensitivity when comparing coupled mitochondria and broken mitochondrial membranes [29]. An obvious proposal, therefore, is that this property of ranolazine may even contribute to its anti-ischaemic action. Thus, it could be envisaged that it would preferentially inhibit wasteful oxygen consumption by uncoupled and broken mitochondria in cells that have been damaged by ischaemia and/or reperfusion, but would hardly affect ATP-generating respiratory activity in cells where the mitochondria remain fully competent. Such compromised cells would also be likely to have lower pH values than normal healthy cells [16] and, thus, more of the compound would be in the protonated and charged form (see above). This also suggests that ranolazine should not suffer from any of the toxicity drawbacks that would normally be associated with a compound that inhibits mitochondrial respiration. Indeed, several studies have indicated that the normal functioning of the heart is unaffected by the drug [5–15], including oxygen consumption under a variety of different perfusion conditions [15]. Thus, it seems very unlikely that there is any significant respiratory inhibition by the drug under normal cellular conditions. In contrast, its effective concentration range with the energetically compromised preparations is close to the effective plasma range seen in antianginal trials [1–4].

This differential potency of ranolazine would also help explain the subtle variations in concentration-dependency of the effect that we often noted with our preparations on a day-to-day basis and on a between-laboratory basis (see legend to Fig. 1) (*i.e.* this would have depended on the quality of the preparations and the degree of coupling achieved).

As already mentioned in the Introduction, the forma-

tion of superoxide and other free radicals is thought to be associated with the mitochondrial damage that occurs during cardiac ischaemia [21–23]. Thus, in the absence of, or lack of sufficient, oxygen as terminal electron acceptor, the electrons generated in the respiratory chain are diverted to form partially reduced free radicals which then, in turn, can attack and damage the respiratory chain enzymes themselves due to peroxidation of lipid components [22–24]. This may also happen on the sudden reintroduction of oxygen into an environment where reducing electrons have built up, as would happen on reperfusion following ischaemia [26]. Complex I, in particular, is thought to be a site of radical production [26], and it has been shown that it can produce, in a NADH-dependent manner, superoxide free radicals by a rotenone-sensitive activity [22]. Therefore, it could be suggested that ranolazine, which clearly acts at a site close to that affected by rotenone, might also help to minimise radical formation in ischaemia and reperfusion. This scenario is supported by the observation that amytal, which similarly inhibits Complex I at a site close to the rotenone site, reduced mitochondrial lipid peroxidation in rabbit hearts reperfused after a period of ischaemia [23]. This effect could not be mimicked by cyanide, and was associated with decreased mean tissue oxygen radical concentration. However, in the study on the inhibitory effects of flunarizine and cinnarizine on Complex I [29] (see above), Veitch and Hue found that, in experiments with submitochondrial particles, flunarizine caused an increase in the NADH-dependent generation of superoxide. This may indicate that the precise site of interaction of the inhibitory compound within the electron sequence of Complex I may dictate whether radical production is inhibited or enhanced. It will, clearly, be important to determine the effects of ranolazine on radical production and lipid peroxidation.

Ranolazine's anti-ischaemic mechanism is thought to involve a stimulation of glucose oxidation at the expense of fatty acid oxidation [13–15]. This would have a number of potential advantages to the ischaemic cell (see Introduction), for instance, increased efficiency of energy production at times of oxygen limitation. It is difficult, at first sight, to see how the effects of ranolazine on the respiratory Complex I could have any relationship to these stimulations of glucose oxidation that have now been observed in several studies on intact perfused hearts [13–15]. Moreover, this effect on glucose oxidation has been observed under several different perfusion conditions, including in normoxia, in low-flow ischaemia, and on reperfusion following global ischaemia [15], (i.e. it does not appear to be substantially affected by the likely changes in mitochondrial energisation status in the tissue, although there was some indication of a modest increase in potency under low-flow ischaemia conditions [15]). This effect also appeared to be evident between 1 and 10 μM and maximal at the latter concentration; as already noted, this range of concentration is the same as the plasma concentration that has been associated with antianginal efficacy in clinical trails [1–4].

Inhibition of Complex I would be expected to increase [NADH] in the matrix which would be expected to decrease flux through pyruvate dehydrogenase [18], the key rate-controlling enzyme in glucose oxidation, which has been shown to be activated by ranolazine [11, 19]. However, an increase in matrix NADH/NAD^+ would also inhibit fatty acid beta-oxidation at the level of the 3-hydroxyacyl CoA dehydrogenase [42], and it may be that it is the relative sensitivity of the carbohydrate and fatty acid pathway enzymes to changes in NADH/NAD^+ that dictates which pathway will predominate. It is, however, already known that ranolazine (up to 1 mM) does not affect carnitine palmitoyl transferase I activity [8], which is thought to be a key rate-controlling enzyme in fatty acid oxidation, and during the course of the present studies we also established (results not shown) that ranolazine (up to 100 μM) does not affect mitochondrial Ca^{2+}-transport, as the matrix concentration of Ca^{2+} is a key determinant of oxidative flux [43]. Thus, at the present juncture, it is perhaps best to propose that it is most likely that ranolazine may have another molecular site of action within the mitochondrial matrix that leads to the observed increases in glucose oxidation. The present work does, however, support this by indicating that ranolazine can interact with processes located within the mitochondrial matrix, and that it may even have greater access to matrix sites under conditions likely to pertain in ischaemia/reperfusion.

The above hypotheses are advanced to try to correlate the presently described inhibitory effects of ranolazine on Complex I observed in the subcellular *in vitro* preparations we have used. This is the first description of a molecular site of action for the drug, with the beneficial effects that have been observed in intact isolated heart and *in vivo* preparations and in clinical trials (see Introduction). In any case, compounds such as ranolazine, with beneficial and inhibitory properties, may be useful tools in elucidating the role of the respiratory Complex I in the context of ischemic and reperfusion damage.

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REFERENCES

1. Cocco G, Rousseau MF, Bouvy T, Cheron P, Williams G, Detry JM and Pouleur H, Effects of a new metabolic modulator, ranolazine, on exercise tolerance in angina pectoris patients treated with beta-blocker or diltiazem. *J Cardiovasc Pharmacol* **20:** 131–138, 1992.
2. Bouvy T, Rousseau MF, Cocco G, Cheron P, William GJ and Detry JMR, Improvement in exercise tolerance and left ventricular filling dynamics in patients with angina pectoris with the novel metabolic modulator, ranolazine. *Acta Cardiol* **48:** 98–99, 1993.
3. Rousseau MF, Visser FG, Bax JJ, Dubrey S, Cocco G, Pouleur H, Harris SR and Wolff AA, Ranolazine: antianginal therapy with a novel mechanism, a controlled comparison versus atenolol. *Eur Heart J* **15** (Suppl.):95, 1994.
4. Smith WB, Chrysant S, Garland WT, Parker TS, Walpole HT, Mokatrin A, Gennevois D and Pepine CJ, A multicenter controlled trial of a novel metabolic active compound (ranolazine) in chronic stable angina patients. *Am Coll Cardiol*, in press.
5. Allely MC, Alps BJ and Kilpatrick AT, The effect of the novel anti-anginal compound RS 43285 on [lactic acid] [K^+] and pH in a canine model of transient myocardial ischaemia. *Biochem Soc Trans* **15:** 1057–1058, 1987.
6. Allely MC and Alps BJ, The effects of the novel antianginal agent ranolazine (i.d.) in a canine model of transient myocardial ischaemia. *Br J Pharmacol* **93:** 246, 1988.

7. Allely MC and Alps BJ, Prevention of myocardial enzyme release by ranolazine in a primate model of ischaemia with reperfusion. *Br J Pharmacol* **99:** 5–6, 1990.
8. Allely MC, Brown CM, Kenny BA, Kilpatrick AT, Martin A and Spedding M, Modulation of α_1-adrenoceptors in the rat left ventricle by ischaemia and acyl carnitines; protection by ranolazine. *J Cardiovasc Pharmacol* **21:** 869–873, 1993.
9. Ferrandon P, Pascal J-C and Armstrong JM, Protective effects of the novel anti-ischaemic agent ranolazine (RS-43285) in the reperfused rat heart. *Br J Pharmacol* **93:** 247, 1988.
10. Brown CM, Clarke B, Dye A, Fraser S, Kenny B, Kilpatrick AT, Patmore L and Whiting R, Pharmacological profile of ranolazine, a metabolic activator active in ischaemia. *Br J Pharmacol* **93:** 248, 1988.
11. Clarke B, Spedding M, Patmore L and McCormack JG, Protective effects of ranolazine in guinea pig hearts during low-flow ischaemia and their association with increases in active pyruvate dehydrogenase. *Br J Pharmacol* **109:** 748–750, 1993.
12. Gralinski MR, Black SC, Kilgore KS, Chou AY, McCormack JG and Lucchesi BR, Cardioprotective effects of ranolazine (RS-43285) in the isolated perfused rabbit heart. *Cardiovasc Res* **28:** 1231–1237, 1994.
13. Collins-Nakai RL, Barr R, Humen D and Lopaschuk GD, Ranolazine stimulates glucose oxidation in isolated working rat hearts. *Can J Cardiol* **9:** (Suppl. E) 33, 1993.
14. Gamble J, Collins-Nakai RL, Barr R, Humen D and Lopaschuk GD, Ranolazine stimulates glucose oxidation in isolated working rat hearts. *Can J Physiol Pharmacol* **72:** (Suppl. 1) 95, 1994.
15. McCormack JG, Barr RL, Wolff AA and Lopaschuk GD, Ranolazine stimulates glucose oxidation in normoxic, ischemic and reperfused ischemic rat hearts. *Circulation*, in press.
16. Lopaschuk GD, Wambolt RB and Barr RL, An imbalance between glycolysis and glucose oxidation is a possible explanation for the detrimental effects of high levels of fatty acids during aerobic reperfusion of ischemic hearts. *J Pharmacol Exp Ther* **264:** 135–144, 1993.
17. Lopaschuk GD, Belke DD, Gamble J, Itoi T and Schoness BO, Regulation of fatty acid oxidation in the mammalian heart in health and disease. *Biochim Biophys Acta* **1213:** 263–276, 1994.
18. Randle PJ, Fuel selection in animals. *Biochem Soc Trans* **14:** 799–806, 1986.
19. Clarke B, Wyatt KM and McCormack JG, Ranolazine increases active pyruvate dehydrogenase (PDH) in perfused normoxic rat hearts. *J Mol Cell Cardiol* **26:** CLXXIV, 1994.
20. McCormack JG and Denton RM, Mammalian mitochondrial metabolism and its regulation. In: *Mitochondria: DNA, Proteins and Disease* (Eds. Darley-Usmar V and Shapira A), pp. 81–112. Portland Press, London, 1994.
21. Shlafer M, Myers CL and Adkins S, Mitochondrial hydrogen peroxide generation and activities of glutathione peroxidase and superoxide dismutase following global ischaemia. *J Mol Cell Cardiol* **19:** 1195–1206, 1987.
22. Narabayashi H, Takeshige K and Minakami S, Alteration of inner-membrane components and damage to electron-transfer activities of bovein heart submitochondrial particles induced by NADPH-dependent lipid peroxidation. *Biochem J* **202:** 97–105, 1982.
23. Cadenas E, Boveris A, Ragan CI and Stoppani OM, Production of superoxide radicals and hydrogen peroxide by NADH-Ubiquinone reductase and Ubiquinol-cytochrome *c* reductase from beef-heart mitochondria. *Arch Biochem Biophys* **180:** 248–257, 1977.
24. Ambrosio G, Zweier JL, Duilio C, Santoro G, Elia PP, Tritto I, Chiariello M and Flaherty JT, Inhibition of mitochondrial respiration and lipid peroxidation in reperfused hearts. *Circulation* **80:** (Suppl. II) 31, 1989.
25. Causey AG, Middleton B and Bartlett K, A study of the metabolism of [U-^{14}C]-3-methyl-2-oxopentoate by rat liver mitochondria using h.p.l.c. with continuous on-line monitoring of radioactive intact acyl-Coenzyme A intermediates. *Biochem J* **235:** 343–350, 1986.
26. McCormack JG and Crompton M, The role and study of mammalian mitochondrial Ca^{2+}-transport and matrix Ca^{2+}. In: *Cellular Calcium: A Practical Approach* (Eds. McCormack JG and Cobbold PH), pp. 345–382. Oxford University Press, Oxford, 1991.
27. Veitch K, Hombroeckx A, Caucheteux D, Pouleur H and Hue L, Global ischaemia induces a biphasic response of the mitochondrial respiratory chain. *Biochem J* **281:** 709–715, 1992.
28. Sherratt HSA, Watmough NJ, Johnson MA and Turnbull DM, Methods for study of normal and abnormal skeletal muscle mitochondria. *Meth Biochem Anal* **33:** 243–335, 1988.
29. Veitch K and Hue L, Flunarizine and cinnarizine inhibit complexes I and II: possible implication for Parkinsonism. *Molec Pharmacol* **45:** 158–163, 1994.
30. Shimomura Y, Kawada T and Suzuki M, Capsaicin and its analogs inhibit the activity of NADH-coenzyme Q oxidoreductase of the mitochondrial respiratory chain. *Arch Biochem Biophys* **270:** 573–577, 1989.
31. Ruzicka FJ and Crane FL, Quinone interaction with the respiratory chain-linked NADH dehydrogenase of beef heart mitochondria. *Biochim Biophys Acta* **223:** 71–85, 1970.
32. Vik SB and Hatefi Y, Inhibition of mitochondrial NADH:ubiquinone oxidoreductase by ethoxyformic anhydride. *Biochem Int.* **9:** 547–555, 1984.
33. Ragan CI, NADH: Ubiquinone oxidoreductase. *Biochim Biophys Acta* **456:** 249–290, 1976.
34. Chudapongse P and Janthasoot W, Mechanism of the inhibitory action of capsaicin on energy metabolism by rat liver mitochondria. *Biochem Pharmacol* **30:** 735–740, 1981.
35. Friedrich T, Van Heek P, Lief H, Ohnishi T, Forche E, Kunze B, Jansen R, Trowitzsch-Kienast W, Hofle G, Reichenbach H and Weiss H, Two binding sites of inhibitors in NADH:ubiquinone oxidoreductase (complex 1). *Eur J Biochem* **219:** 691–698, 1994.
36. Harpey C, Clauser P, Labrid C, Freyria J-L and Poirier J-P, Trimetazidine, a cellular anti-ischemic agent. *Cardiovasc Drug Rev* **6:** 292–312, 1989.
37. Fantini E, Demaison L, Sentex E, Grynberg A and Athias P, Some biochemical aspects of the protective effect of trimetazidine on rat cardiomyocytes during hypoxia and reoxygenation. *J Mol Cell Cardiol* **26:** 949–958, 1994.
38. Cheng YC and Prusoff WH, Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymic reaction. *Biochem Pharmacol* **22:** 3099–3108, 1973.
39. Filser M and Werner S, Pethidine analogues, a novel class of potent inhibitors of mitochondrial NADH:ubiquinone reductase. *Biochem Pharmacol* **37:** 2551–2558, 1988.
40. Javitch JA, D'amato RJ, Strittmatter SM and Snyder SH, Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetra hydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proc Natl Acad Sci USA* **82:** 2173–2177, 1985.
41. Ramsay RR, Salach JI and Singer TP, Uptake of the neurotoxin 1-methyl-4-phenylpyridine (MPP^+) by mitochondria and its relation to the inhibition of the mitochondrial oxidation of NAD^+-linked substrates by MPP^+. *Biochem Biophys Res Commun* **134:** 743–748, 1986.
42. Latipaa PM, Karki TT, Hiltunen JK, Hassinen IE, Regulation of palmitoyl carnitine oxidation in isolated rat liver mitochondria. *Biochim Biophys Acta* **875:** 293–300, 1986.
43. McCormack JG, Halestrap AP and Denton RM, Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol Rev* **70:** 391–425, 1990.