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EFFECTS OF BEPRIDIL[*] ON MITOCHONDRIAL ATPase REACTIONS

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Abstract—Bepridil has a biphasic effect on oligomycin sensitive ATPase[†] activity. At pH 8 and at concentrations below 12 μM, it inhibits ATPase activity, whereas at higher concentrations it stimulates this activity. When the pH is either more acid or more alkaline, this dual effect disappears. At pH 7, only the inhibitory effect remains but at pH 9, the stimulatory effect is observed. Inhibition is uncompetitive, with a K_i of the order of 30 μM.

Even at low concentrations, bepridil reactivates oligomycin-inhibited OS-ATPase. It does not affect soluble ATPase (F1), except if the enzyme has previously been treated by its natural inhibitor, in which case bepridil potentiates the inhibitory effect. Bepridil does not seem to have the same influence on all ATP-driven mitochdrial membrane reactions: thus, it inhibits the reverse electron flow between the succinate and NAD, but hardly alters the transhydrogenase NADH/NADP reaction. The possibility is discussed that bepridil may have a different mechanism of action depending on whether or not it is protonated.

Bepridil is designed for the treatment of angina pectoris. Among other properties, it improves coronary blood flow by its direct vasodilatory effect, restricts myocardial oxygen consumption and reduces tachycardia. These properties have been observed under three conditions; in intact heart, denervated heart or after propranolol administration [1]. A non-competitive type of antagonism in relation to papillary muscle contraction was noted between bepridil on the one hand and Ca^{2+} and isoprenaline on the other. These results may be attributed to the action of bepridil on cell membranes [2, 3]. In our experiments, we showed that bepridil also affected mitochondrial function [4]. It inhibited oxidative phosphorylation of NAD-linked substrates. Such inhibition involved parallel reduction in oxygen consumption and ATP formation without changing the P/O ratio. On the other hand, bepridil also uncoupled phosphorylation linked to succinate oxidation. Both the above effects might be due to the drug's degree of protonation along the mitochondrial membrane, which generates a pH gradient. The applications of Mitchell's chemiosmotic theory [5] have demonstrated that the cystolic side of the mitochondrial membrane is more acid than the matricial side [6–8]. Moreover, unlike NADH dehydrogenase, succinate dehydrogenase does not appear to be involved in proton transport across the inner mitochondrial membrane [9]. This implies that phosphorylation would be inhibited by bepridil in an acid medium, and uncoupled in a base medium.

The effect of bepridil on oxygen consumption seems to result from the drug's influence on ATP-linked reactions. The results given below show that it definitely alters ATPase isolated from mitochondria.

METHODS

Pig heart mitochondria were prepared according to the method of Crane *et al.* [10], as described by Gautheron *et al.* [11]. The energy-linked reactions, NAD^+ reduction by succinate [12] and the $NADH/NADP^+$ transhydrogenation reaction [13], both of them ATP-linked, were studied in mitochondrial sub-particles, isolated according to Hansen and Smith [14]. Oligomycin-sensitive ATPase (OS-ATPase) was isolated from mitochondrial sub-particles by Lubrol and Triton × 100 fractionation according to Maîrouch and Godinot [15]. Soluble

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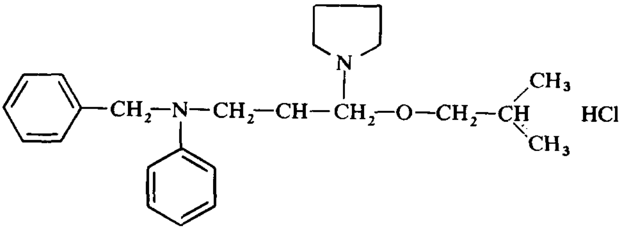
[*] CERM patents: FR 720767 and US 3962238. [(*N*-benzyl *N*-phenyl amino)-1 isobutoxy-3-propyl]-2 pyrolidine, hydrochloride.



[†] OS-ATPase, oligomycin sensitive ATPase.

ATPase (F1) was purified by treating mitochondrial sub-particles with chloroform and subsequent precipitation with ammonium sulphate [16]. ATPase and ATP-driven reactions are described in the table and figure legends. Proteins were measured by the method of Lowry *et al.* [17].

RESULTS

The nature of the change in OS-ATPase activity caused by bepridil depends on the pH (Fig. 1). At pH 8, the drug's effect was biphasic: at concentrations below 12 μM, it inhibited mitochondrial membrane ATPase activity; on the other hand, at concentrations above 12 μM, the inhibitory effect was reversed and activity rose steadily, reaching twice the control level at 120 μM bepridil. When the pH was more acid, this dual effect disappeared and inhibition occured (pH 7). With a more alkaline pH, the effect was replaced by activation (pH 9). In both cases, there was saturation at approximately 50 μM bepridil.

The results in Fig. 2 show that inhibition of ATPase activity by bepridil was uncompetitive. This graph also enabled us to calculate the K_m and V_m for OS-ATPase in relation to the substrate (ATP), 150 μM and 4.2 μmoles/min/mg protein, respectively.

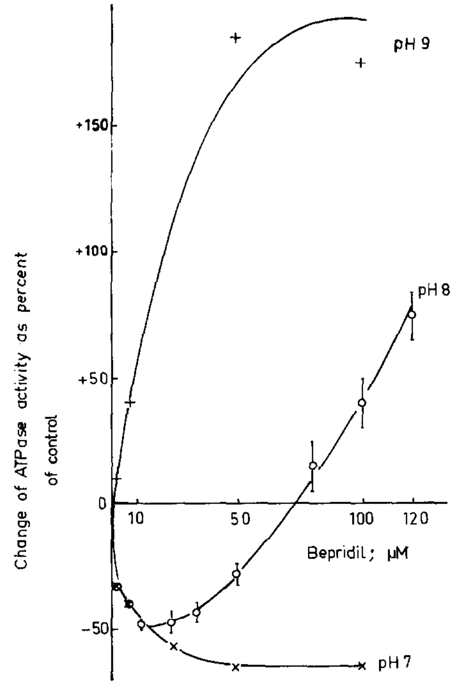


Fig. 1. Effect of bepridil on OS-ATPase activity. Results are expressed in relation to the control value obtained in the absence of bepridil. Enzyme suspensions (10 μl) containing from 0.3 to 0.4 mg protein/ml were added to a medium composed as follows (final volume: 0.65 ml): 50 mM Tris-maleate buffer (pH indicated on curve), 5 mM $MgCl_2$, 3 mM ATP, 4 mM PEP as Na^+-salt, 0.4 mM NADH, 30 units lactic dehydrogenase and 30 units pyruvate kinase. The ATP hydrolysis reaction was followed by the absorbance of NADH at 340 nm. Ethanol bepridil solution (5 ml) (final concentration given in abscissa) were added to the preparation, ethanol concentration was 80 μmoles in the medium. Vertical bars represent standard deviation of 10 experiments.

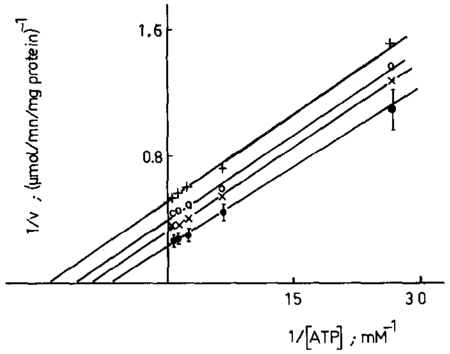


Fig. 2. Lineweaver–Burk plot showing the kinetics of the inhibition of OS-ATPase activity by bepridil. Bepridil concentrations were, respectively, 0 (●), 7.5 (×), 15 (○) and 30 μM (+). Experiments were carried out as described in Fig. 1, except that the ATP concentration was changed to obtain the values given in abscissa, and pH 8.

The constant for ATPase inhibition by bepridil was 22 μM (Fig. 3), calculated from the slope of the Dixon plot.

Figure 4 shows that OS-ATPase activity was inhibited by oligomycin, and also how this inhibition was affected by bepridil. In the absence of bepridil, 80 per cent inhibition was observed at an oligomycin concentration of 3.4 μg/mg protein. Addition of 120 μM bepridil changed the degree of OS-ATPase inhibition by oligomycin. These results seem to indicate a certain amount of competition between bepridil and oligomycin, since raising the oligomycin concentration lowered the activation produced by high bepridil concentrations. Nevertheless, the results in Fig. 5 show that the inhibitions obtained with low bepridil concentrations and oligomycin, respectively, were not cumulative; on the contrary, the opposite was the case, since we observed a reduction in the inhibition caused by oligomycin.

Appropriate treatment with trypsin raised OS-ATPase activity following destruction of the polypeptide which is the natural ATPase inhibitor [18].

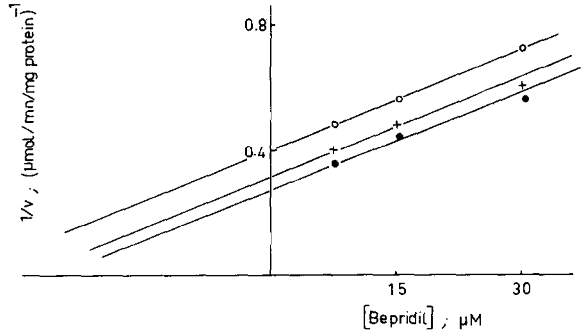


Fig. 3. Determination of the inhibition constant (K_i) for OS-ATPase inhibition by bepridil. Dixon plot; the inverse of the enzyme reaction rate was plotted against the bepridil concentration, for three ATP concentrations: 0.75 (●), 0.375 (+) and 0.15 mM (○). Experimental conditions are given in the legend to Fig. 1.

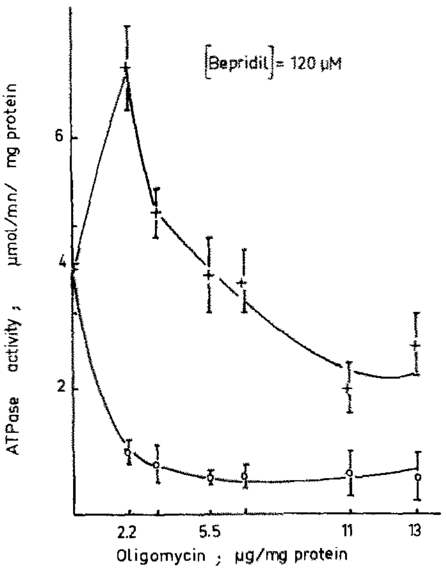


Fig. 4. Interactions between the effects of bepridil and oligomycin on OS-ATPase activity. This activity was measured in the presence of different oligomycin concentrations, plotted in abscissa in the absence (○) or presence (+) of 120 μM bepridil. Activity of 3.9 ± 0.7 μmoles/min/mg protein is the control experiment, without oligomycin or bepridil. Determinations were made as in Fig. 1. pH 8 vertical bars represent standard deviations, number of experiments was 10.

Since this treatment rendered ATPase insensitive to bepridil (results not shown), we attempted to study the effects of bepridil on the interaction of soluble ATPase (F1) with its natural inhibitor. Table 1 shows that bepridil has no direct effect on this ATPase. Treatment of F1 by the ATPase inhibitor reduced enzyme activity. In addition, bepridil was seen to potentiate this reduction. This inhibitory effect on soluble ATPase whose structure is necessarily changed by the inhibitor suggests that bepridil might inhibit certain energy-driven reactions requiring the presence of F1 in the mitochondrial membrane [19].

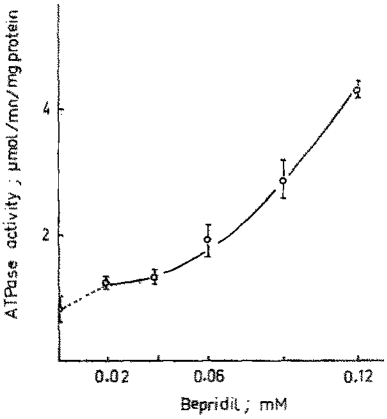


Fig. 5. Interactions between the effects of bepridil and oligomycin on OS-ATPase activity. Same experiments as in Fig. 4, except that activity was measured for a fixed oligomycin concentration of 3.4 μg/mg protein and for various bepridil concentrations.

In this connection we demonstrated that NAD reduction by the succinate, which required ATP, was inhibited by bepridil (Table 2). On the other hand, the transhydrogenase reaction was almost unaffected when subparticles were treated with the same bepridil concentrations as those which inhibited NAD reduction.

DISCUSSION

The observation that bepridil exerts opposite effects on OS-ATPase activity, depending on the pH, suggests that OS-ATPase responded differently to bepridil depending on whether it was protonated (BH^+) or dissociated (B). On the basis of the product's dissociation constant (pK_a : 6.3), it may be concluded that the acid form is inhibitory, whereas the base form is an activator. Membrane ATPase does not therefore appear to have the same affinity for these two forms of bepridil. For both of them, the

Table 1. Effect of bepridil on interaction between soluble ATPase (F1) and the ATPase natural inhibitor

	(Bepridil): μM						
Addition	0	8	20	40	60	80	120
0*	51	59.5	52	54	51.5	52	52
ATPase inhibitor†	29		17	13.2	12.4		
	−45%		−68%	−75%	−77%		

* Figures in the table express ATPase activity in μmoles of ATP hydrolyzed per min and per mg protein at room temperature. Enzyme solution (10 μl) was added to a final volume of 0.65 ml medium containing 50 mM Tris-maleate buffer (pH 8), 5 mM $MgCl_2$, 3 mM ATP, 0.4 mM NADH, 4 mM PEP and 30 units LDH and PK, respectively, with and without 5 μl of an alcoholic solution of bepridil at the indicated concentration. The reaction rate was determined by the decrease in absorbance at 340 nm.

† According to Horstman and Racker [25], ATPase at 1–2 i.u. was incubated for 20 min in a final volume of 0.5 ml containing 0.4 μg of ATPase inhibitor, in the presence of 0.5 mM ATP, 10 μM Tris-sulphate and 0.15 M sucrose (pH 6.7). After incubation, 50 μl were removed for ATPase activity determinations as specified above.

Table 2. Effect of bepridil on ATP-driven reaction

	(Bepridil: μM)					
Reaction	0	0.4	1.6	4	12	24
NAD succinate-linked reduction*	68.4	61.7 −10%	58.7 −14%	56.6 −17%	48.6 −29%	33.4 −49%
NADH/NADP transhydrogenation†	82.5	80.4 −2.5%	82.5 0	76.6 −7%	74.3 −10%	70 −15%

* ATP-driven succinate linked NAD$^+$ reduction: 1 mM ATP was added to a reaction medium, maintained at 30°C, containing, in a final volume of 3 ml, 50 mM Tris-HCl, (pH 7.5) 0.25 mM sucrose, 6 mM $MgCl_2$, 10 mM succinate, 1 mM NAD, 1 mM KCN and mitochondria subparticles (0.4–0.5 mg) protein. NAD reduction was observed spectrophotometrically at 340 nm.

† ATP-driven transhydrogenase: 1 mM ATP was added to a reaction medium, maintained at 30°C, containing, in a final volume of 3 ml, 50 mm Tris–HCl (pH 7.5), 0.25 M sucrose, 80 mM ethanol, 6 mM $MgCl_2$, 100 μg alcohol dehydrogenase, 0.4 mM NADP$^+$ and 0.15 mM NADH. NADPH formation was observed spectrophotometrically at 340 nm.

Hendersen–Hasselbalch equation enables calculation of the concentrations with the maximum effect on OS-ATPase activity, as follows: at pH 7, (BH$^+$) = 8 μM and (B) = 42 μM, for a total concentration of 50 μM— in this case, only the inhibitory effect is observed, and at pH 9, the BH$^+$ concentration is negligible, which explains the predominance of the activating effect.

The effects of the pH suggest that the mode of action of bepridil is due to its interference with proton movement in the OS-ATPase membrane sector. This conclusion is corroborated by the present results for interaction between the effects of bepridil and oligomycin. Oligomycin inhibits ATPase activity by blocking proton translocation across the mitochondrial membrane [20]. Our data suggest that interference occured between the BH$^+$ form and the oligomycin action site. Thus, at pH 8, with a bepridil concentration of 120 μM (i.e. 2.35 μM BH$^+$ and 117.65 μM B), the inhibitory effect of oligomycin was abolished and some enzyme activation was even observed. Such activation may reach the values found in the absence of oligomycin (Fig. 1). In addition, the effect of oligomycin diminished at inhibitory bepridil concentrations.

The results suggest that bepridil and certain ionophores like valinomycin and nigericin might have similar functions. In this respect an ionophores/oligomycin antagonism has been demonstrated with regard to mitochondrial ATPase activity [21]. However, the antagonism was observed on sonicated mitochondria but did not occur with purified OS-ATPase. This differs from the present results, inasmuch as the antagonism was demonstrated with purified OS-ATPase. Another difference was that the authors cited above [21] found that valinomycin had no direct effect on mitochondrial ATPase, whereas we observed that bepridil did have such an effect.

Kinetic studies showed that the drug did not directly influence the ATPase catalytic site, a conclusion supported by the fact that bepridil failed to modify the activity of the soluble ATPase (F1). On the other hand, bepridil potentiated the inhibitory effects on F1 of the ATPase inhibitor. This potentiation seems to us very important, since ATPase-inhibitor interaction is thought to regulate many different ATP-driven reactions [22–24]. Such potentiation might partly explain the effect of bepridil on energy linked reactions because the drug does not behave exactly like the natural ATPase inhibitor (Table 2); for instance, it did inhibit NAD reduction by succinate but not the transhydrogenase reaction.

From the present results, it may justifiably be concluded that bepridil probably has three action sites: two of them are in the membrane and the nature of their mechanism of action depends on whether or not the bepridil is protonated. The third site is located on F1, the protein carrying the ATPase activity. Bepridil binding to this potentiates the effect of the natural ATPase inhibitor by reducing the activity of the ATP-linked reactions.

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