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EFFECTS OF BEPRIDIL* ON Ca^{2+} UPTAKE BY CARDIAC MITOCHONDRIA

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Abstract—Isolated rat heart mitochondria accumulate large amounts of Ca^{2+} at the expense of respiration-linked energy or of that provided by the hydrolysis of ATP by the mitochondrial ATPase. At concentrations below 10 μM bepridil has no effect on the first mechanism but inhibits the second. At higher concentrations bepridil depresses both. At low concentrations bepridil decreases proton influx into mitochondria in ADP-stimulated respiration while it has no effect on proton ejection in Ca^{2+}-stimulated respiration. A preliminary study shows that bepridil inhibits ATP hydrolysis linked to Ca^{2+} absorption by mitochondria. The calcium antagonists verapamil, nifedipine and diltiazem exhibit none of these effects.

Bepridil is a new antianginal and antiarrhythmic agent. It depresses cardiac contraction and reduces oxygen consumption of the heart [1, 2]. At cellular level the drug has been shown to act like calcium antagonists [3–6]. However, when studies were carried out on subcellular preparations, these compounds were found to behave differently. Nifedipine and diltiazem did not alter the calcium binding to cardiac sarcolemma from guinea pigs, whereas verapamil and, to a lesser degree, bepridil decreased the binding to the low-affinity calcium sites [7, 8]. Bepridil differs from verapamil in many respects. Thus bepridil is a less potent inhibitor than verapamil of the slow inward current in guinea pig myocardium [3] and in vascular smooth muscle of dog coronary artery [5]. In addition, bepridil decreases the duration of the induced slow action potentials of frog skeletal muscle at a concentration far below that of verapamil. At the same time both compounds decrease to the same extent the amplitude of those potentials [9]. Bepridil inhibits contractions nearly completely while slow action potentials still occur, whereas with verapamil the two parameters are closely correlated [3]. Accordingly, a second intracellular action site for bepridil was suggested [3]

Very recently it was shown that myocytes can concentrate bepridil up to 1.2 mM when the extracellular concentrations is 10 μM and in the same study actin was identified as a possible intracellular site of action [10]. However, other internal targets cannot be excluded, since mitochondria and sarcoplasmic reticulum react to bepridil below 10 μM. We have shown that bepridil alters the activities of Ca-dependent ATPase from sarcoplasmic reticulum [11] and of mitochondrial ATPase [12]. The inhibitor effect in the latter case can explain the decrease in oxidative phosphorylation produced by the drug [13].

Treatment with calcium antagonists decrease the myocardial lesions in hamsters of the myopathic line while improving the respiratory control of mitochondria isolated from the same animals [14, 15]. These consequences may be related directly or indirectly to their protective effect against mitochondrial damage induced by metabolites which accumulate in the ischemic myocardial cells, e.g. Ca^{2+} [14, 16]. The results of the present study show that bepridil, unlike verapamil, diltiazem and nifedipine, inhibits Ca^{2+} influx into mitochondria when the energy needed for this process is provided by ATP. On the other hand none of the above compounds affect the process driven by the energy from oxidation.

MATERIALS AND METHODS

Bepridil, nifedipine, verapamil and diltiazem were provided by Organon Laboratories Ltd, Bayer Pharma, Biosedra and Dausse, respectively. All other chemicals were of analytical reagent grade.

Mitochondria from hearts of Wistar rats weighing 300–350 g were isolated according to the method of Tyler and Gonze [17]. This preparation gave well-coupled mitochondria as indicated by the RCR of 6 and the ADP/o ratio of 2 for succinate as substrate.

Protein content was determined according to the Lowry method [18].

Oxygen consumption was measured with a Clark electride using a Gilson oxygraph (IC-Oxy). The reaction vessel was placed in a water bath at 30° and contained the following, in a final volume of 1.6 ml: 130 mM KCl, 3 mM Pi, 6 mM succinate, 2.4 μM rotenone, 0.6–1 mg mitochondrial protein and 3 mM Hepes buffer pH 7.2. Proton movement in the same solution was followed by a pH electrode (Ca 14//02, Philips) connected to a Philips ionometer (PW 94/4); according to Vercesi *et al.* [19].

Calcium uptake was determined by a specific electrode (IS-561 Ca type, Philips) according to Simon *et al.* [20], as follows; a final concentration of 4 mM succinate or 0.2 mM ATP was added to 5 ml of 3 mM

* B [(2-methylpropoxy) methyl)]-*N*-phenyl-*N*-phenylmethyl-1-pyrrolidine-ethanamine (ORG 5730).

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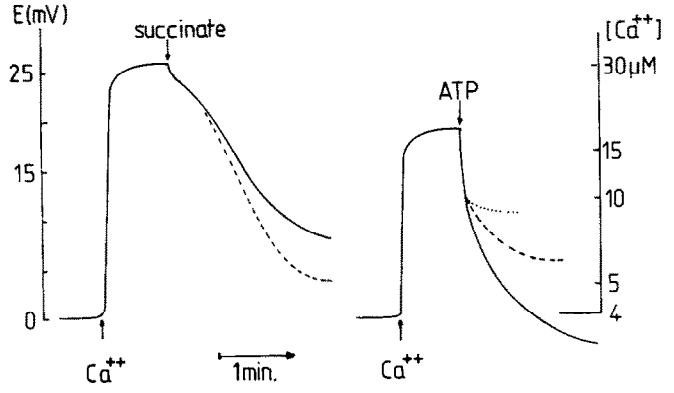

Fig. 1. Ca^{2+}-uptake by mitochondria from rat heart. The free calcium concentration in the medium was determined by a specific electrode as described under Materials and Methods. The experimental conditions were as follows: 5 ml of 3 mM Hepes buffer pH 7.2, containing 130 mM KCl, 2 mM glutamate, 2 mM Pi, 1.5 mg of mitochondrial protein and 4 μM rotenone, were preincubated at 30° for 5 min, then 10 μl containing 200 or 100 nmoles of $CaCl_2$ were added to the succinate experiment (left) and to the ATP experiment (right) respectively. Calcium uptake was initiated by adding succinate 4 mM or ATP 0.2 mM final concentration. The solid lines represent the control experiments, the broken lines the experiments in the presence of 4.25 μM bepridil. The dotted line (the ATP experiment) represents the experiments without mitochondria, which was necessary to evaluate the amount of Ca^{2+} remaining as Ca-ATP complex, the same recording was obtained when the experiment with mitochondria was done in the presence of oligomycin (1 μg/mg protein).

Hepes buffer pH 7.2 containing 130 mM KCl, 2 mM glutamate, 2 mM Pi, 4 μM rotenone and 1–1.5 mg of mitochondrial protein, at 30°. A decrease in free (Ca^{2+}) was followed by a decline in the electrode potential. The electrode response in mV to the free Ca^{2+} is given by the following experimental equation: $E = -36 + 28 \log (Ca^{2+})$ where the Ca^{2+} concentration is expressed as nmoles of Ca^{2-} in the medium.

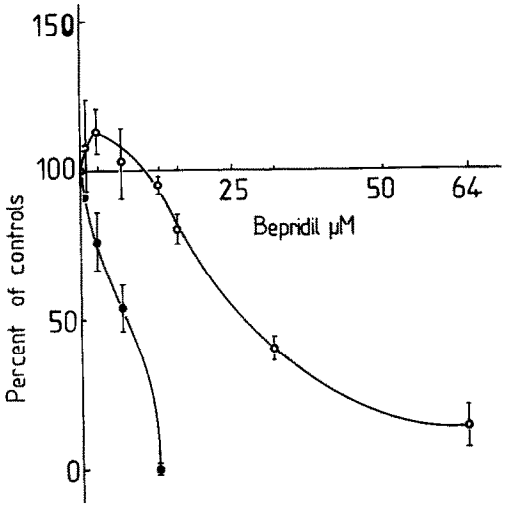

Fig. 2. Effect of bepridil on Ca^{2+} uptake by rat heart mitochondria. Results are expressed as (%) of control obtained in the same conditions as those of Fig. 1 except that the Ca^{2+} concentration was the same in all experiments: 300 nmoles $CaCl_2$ in the medium (●—●) ATP as the source of energy, (○—○) succinate as the source of energy. Values are means ± S.E.; the number of experiments was at least 5. The control values were 296 ± 26 for 7 experiments with succinate and 304 ± 19 nmoles/min/mg protein for 5 ATP experiments.

In the presence of ATP, the response was weaker, due to the formation of an ATP-Ca^{2+} complex. In that case, the standardization gave a linear relation of 0.37/1 nmoles between free Ca^{2+} and total Ca^{2+}. The rate of Ca^{2+} uptake by mitochondria was calculated from the recording of the electrode response by subtracting free Ca^{2+} measured 30 sec after the addition of substrate from the initial Ca^{2+}. The electrode technique was used by many authors to study the energy-linked influx of Ca^{2+} into mitochondria [21, 22]. The kinetic parameters V_m and K_m we have obtained (see Table 1) are very close to those reported by the literature [22, 23].

Mitochondrial ATPase activated following Ca^{2+} accumulation by mitochondria was measured by recording directly the rise in acidity.

RESULTS AND DISCUSSION

Uptake of Ca^{2+} by mitochondria. Mitochondria accumulate Ca^{+} from the surrounding medium until the extramitochondrial free Ca^{2+} concentration is approximately 1 μM [24]. This process is induced either by energy from respiration or from ATP hydrolysis [25, 26] and depends on the presence of a specific carrier [27, 28]. The results presented in Figs. 1 and 2 show that at concentration below 10 μM, bepridil did not affect the binding of Ca^{r+} to its carrier but rather the energy supply mechanism involved in the Ca^{2+} influx into mitochondria. When the energy was supplied by respiration, succinate oxidation in our experiment, bepridil tended to increase the rate of Ca^{2+} uptake. On the other hand, when the ATP was used to promote the Ca^{2+} influx, bepridil completely abolished the process. The specificity of the drug, at low concentrations, in inhibiting the ATP-linked calcium uptake into mitochondria was confirmed by the experiment shown in Fig. 3.

Table 1. Effects of bepridil on the kinetic parameters* V_m and K_m for Ca^{2+} flux into mitochondria

	Succinate oxidation		ATP hydrolysis	
Source of energy	V_m	K_m (μM)	V_m	K_m (μM)
Control	670	25	700	15
Bepridil				
4.25 μM	700	25	465	22
8 μM	800	25	400	22

* Both parameters were calculated from a Lineweaver–Burk plot for (Ca^{2+}) = 100, 80, 60 and 20 μM in the succinate case and 37.5, 30, 22.5, 15 and 7.5 μM in the ATP experiments (as nmoles/min/mg protein).

At 10 μM bepridil almost abolished Ca^{2+} influx in the presence of ATP. Addition of succinate restored the influx to its control level. At higher concentration the drug, even in the succinate case behaved as an inhibitor. The question is whether these concentrations of free bepridil do occur within the cell? As a matter of fact the most accumulated drug in the isolated myocytes was shown to bind to actin [10]. The data in Table 1 indicate that bepridil did not interact with the binding of Ca^{2+} (K_m is constant). On the other hand, the changes in the V_m may reflect an alteration in the proton movement across the mitochondrial membrane [19, 12].

ATPase-linked reactions. Addition of ADP or Ca^{2+} stimulates oxygen consumption by mitochondria. This stimulation is accompanied by absorption, in the ADP case, or ejection, in the Ca^{2+} case, of a known amount of H^+. The oxygen consumption then returns to the original state 4 rate. The absorption of H^+ in the ADP-induced state 3 is specific to the mitochondrial ATPase. On the other hand, when Ca^{2+} is transported into respiring mitochondria electrophoretically in response to the negative-inside membrane potential (ψ) established by electron-transport-driven vectorial H^+ ejection, the mitochondrial ATPase is not involved [28]. Figure 4 shows that bepridil has a very slight effect on H^+ ejection during stimulation of oxygen consumption by Ca^{2+} while it inhibits H^+ absorption induced by ADP addition. This inhibition was parallel to the decrease in oxygen consumption and in ATP synthesis [13]. From this and the direct action of bepridil on isolated mitochondrial ATPase [12], we may conclude that bepridil is an inhibitor of ATPase-linked reactions: such as Ca^{2+} accumulation in mitochondria. Moreover the inhibition of Ca^{2+} uptake by mitochondria (Fig. 2) is related to the inhibition of ATP hydrolysis as can be seen in Fig. 5. Interestingly the inhibition of Ca-activated mitochondrial ATPase by bepridil (Fig. 5) has the same profile as that of isolated ATPase at pH 7 [12]; i.e. inhibition reaches 50% of control at bepridil concentration around 10 μM.

The other calcium antagonists (verapamil, nifedipine and diltiazem), known for their therapeutic similarity to bepridil, did not affect calcium uptake by heart mitochondria (results not shown). But this did not exclude a possible role of these drugs in controlling the calcium transport across the mitochondria. Indeed, diltiazem has been shown to inhibit Ca^{2+} efflux from heart mitochondria by the Na/Ca exchange pathway [29].

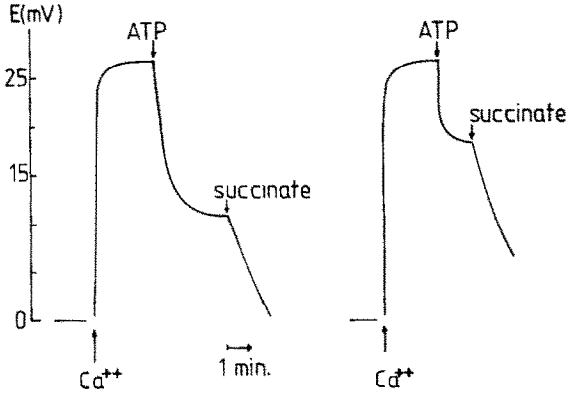

Fig. 3. Succinate-dependent Ca^{2+}-uptake in the presence of ATP. Control experiment (left) and the experiment in presence of 10 μM bepridil (right). The experimental conditions are reported in the legends of Fig. 1 except that the added $CaCl_2$ was the same (200 nmoles) for the experiments with or without bepridil.

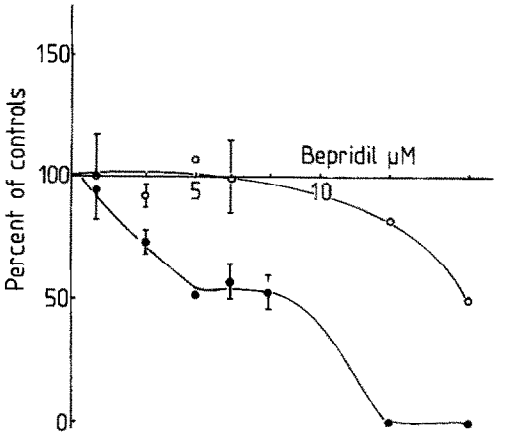

Fig. 4. Effect of bepridil on proton efflux during the stimulation of mitochondrial oxygen consumption by Ca^{2+} (○—○) or on proton uptake when oxygen consumption was stimulated by ADP (●—●). In the two cases mitochondria were respiring on succinate as substrate Ca^{2+} and ADP concentrations were 0.2 mM. The control values for 3 different experiments were 221 ± 20 and 391 ± 13 ng ions/min/mg protein for ADP and Ca^{2+} experiments respectively.

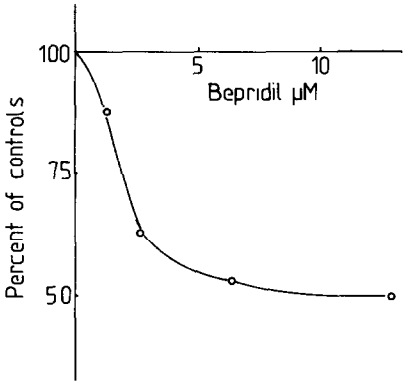

Fig. 5. Effect of bepridil on ATP hydrolysis induced by Ca^{2+} flux into mitochondria. The reaction rate was measured in terms of rise in acidity due to $ATP^{4-} + H_2O \rightleftarrows ADP^{2-} + HPO_4^{2-}\} + \{H^+$. The control values for 7 different experiments was 150 ± 20 ng H^+/min/mg protein.

To explain the full effect of bepridil an intracellular action was suggested [3]. This suggestion is supported by the ability of the drug to enter the cardiac cell [10] and to alter the function of ioalated mitochondria [13] in such a manner as to spare ATP. This property might be very important when, if oxygen is lacking, calcium fluxes massively into and accumulates in mitochondria thus depleting the cell in ATP. Whether this is what actually happens within the cell remains to be demonstrated.

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