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EFFECTS OF BEPRIDIL* ON THE Ca-DEPENDENT ATPase ACTIVITY OF SARCOPLASMIC RETICULUM

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Abstract—The changes in Ca^{2+}-ATPase (Ca^{2+}-stimulated, Mg^{2+}-dependent ATP phosphohydrolase, EC 3.6.1.3) caused by Bepridil depend on whether or not this enzyme is purified. Bepridil has a biphasic effect on the ATPase activity of intact sarcoplasmic reticulum. At concentrations below 10 μM, it stimulates this activity and inhibits it at higher concentrations.

On the other hand, when the Ca^{2+}-ATPase is purified, Bepridil only has an inhibitor effect.

Preliminary study of the action of Bepridil on Ca^{2+} chelating agents indicates that in the absence of Ca^{2+} ions, this drug forms soluble complexes with EDTA like chelating agents; conversely such complexes are only formed with EGTA like chelating agents in the presence of calcium. This last property is accompanied by the release of calcium ions.

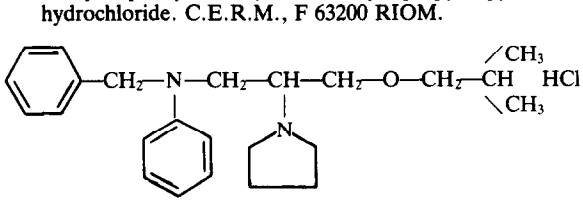
There is reason to think that Bepridil might have opposite effects, depending on whether the calcium complexes are localised within the vesicle of the sarcoplasmic reticulum or on the active site of the corresponding ATPase.

Bepridil is designed for the treatment of angina pectoris because it improves coronary circulation by direct vasodilation, and reduces oxygen consumption and tachycardia. Bepridil is not a beta-blocker, since these properties are not modified by prior administration of propanolol or cardiac muscle denervation [1]. In addition, Bepridil does not competitively inhibit the effects of isoprenalin [2]. Lastly, it does not affect the trachea muscle relaxation caused by isoprenalin, thus excluding any direct interaction between Bepridil and adenylate cyclase [2].

Subcellular and molecular studies have shown that Bepridil alters membraneous functioning in the cardiac cell by inhibiting oxidative phosphorylation of mitochondria isolated from cardiac muscle [3]. Such inhibition is due to the interference of Bepridil with proton movement across mitochondrial ATPase [4].

Bepridil has also been shown to block the slow channels of plasma membrane [5]. The drug's properties seem to result from its action on ion transport by cell membranes. The results of the present study clearly show that this is true of the sarcoplasmic reticulum.

* C.E.R.M. patents: FR 720767 and US 3962238. (*N*-benzyl *N*-phenyl-amino)-1-isobutoxy-3-propyl-2-pyrolidine hydrochloride. C.E.R.M., F 63200 RIOM.



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Abbreviations used: EGTA, ethyleneglycol-bis-(β-amino-ethyl ether) *N,N'*-tetra-acetic acid.

MATERIALS AND METHODS

Approximately 400 g of Albino rabbit white muscle was used to prepare sarcoplasmic reticulum and calcium-dependent ATPase according to MacLennan [6]. The fractions used were R1, corresponding to the intact sarcoplasmic reticulum which MacLennan called washed R1, and R3, the ATPase stripped of many of the other proteins involved in calcium binding by the reticular membrane.

ATPase activity was measured by Pullman and Monroy's ATP regeneration system [7], under the experimental conditions described in the figure legends. Ca^{2+}-dependent ATPase activity was obtained by subtracting total activity from 'basal' EGTA-independent activity [8]. The free calcium concentration in the medium was estimated by a calcium-specific electrode, according to the improved technique of Simmon *et al.* [9]. Protein composition was determined by SDS polyacrylamide gel electrophoresis according to Weber and Osborn [10], and proteins were measured by the method of Lowry *et al.* [11].

RESULTS

Figure 1 shows the electrophoretic pattern of the R1 and R3 fraction used to study ATPase activity. The left part of the figure indicates the protein composition of the intact sarcoplasmic reticulum. The molecular weights of proteins 1, 2, 3 and 4 were 105,000, 44,600, 41,000 and 6,000, respectively. Of these proteins, 1 corresponds to ATPase [6, 12], and 2 and 3 correspond to calsequestrin and high affinity Ca^{2+} binding protein, respectively [13–15]. Protein 4 was characterised by MacLennan *et al.* as a proteolipid [16]. During preparation of the R3 fraction, its protein 1 content was enriched compared to that

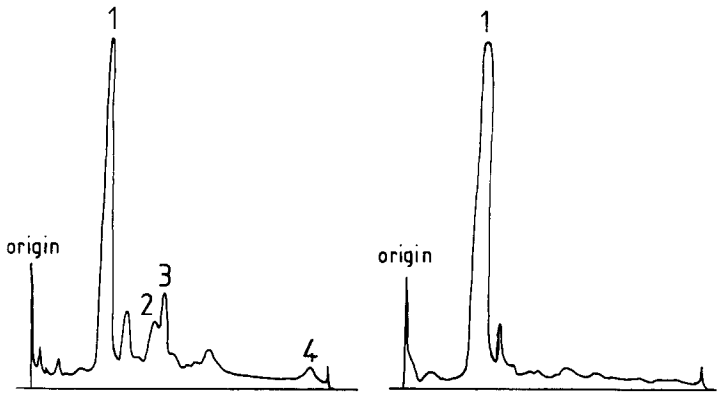

Fig. 1. Gel electrophoresis densitometric tracing of R3 (right part) and washed R1 (left part) fractions obtained by MacLennan's method [6]. Proteins were denatured by heating to 90° for 3 min in a medium containing 2% β-mercaptoethanol and 2% sodium dodecyl sulfate (SDS). Electrophoresis was carried out on 7% polyacrylamide gel columns of 12 × 0.6 cm in a 0.1 M sodium phosphate buffer, pH 7.2, containing 2% SDS and 2% β-mercaptoethanol (8 mA/column). Protein bands were stained with Coomassie blue.

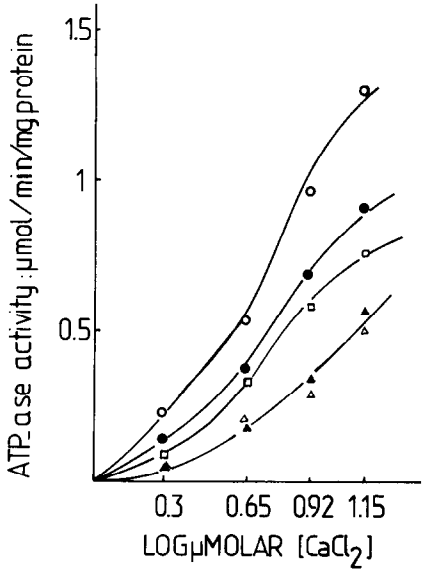

Fig. 2. Effect of Bepridil on the calcium-dependent ATPase activity of intact sarcoplasmic reticulum (washed R1). Measurements were made at room temperature (approximately 20°) in a spectrophotometer cuvette at 340 nm. The reaction medium of 0.65 ml contained 50 mM Tris–HCl buffer, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.4 mM NADH, 4 mM PEP, 30 units lactic dehydrogenase, 30 units pyruvate kinase and 50 µg protein from the enzyme suspension. The reaction was started by adding 3 mM ATP. To obtain 'basal' ATPase activity, 50 µM EGTA was added to the medium. This activity was subtracted from the total activity obtained after adding calcium, whose concentrations are indicated in abscissa. The free calcium ion concentration was calculated on the basis of 0.21 μM^{-1} as the apparent binding constant of EGTA for Ca^{2+}. We have calculated this constant from an experiment made in the same medium used for the ATPase study. □ control activity without Bepridil, ○ 2.5 µM Bepridil, ● 7 µM Bepridil, ▲ 55 µM Bepridil, △ 110 µM Bepridil.

of the R1 fraction (85% vs 55%). In addition, R3 specific activity was nearly twenty times that of R1.

ATPase activity in sarcoplasmic reticulum is calcium dependent. Accordingly this activity seemed to reach its maximum for calcium concentrations of about 15 µM. $Ca^{2+}{}_{0.5}$ (the concentration necessary to obtain half maximum velocity) was around 3 µM (Fig. 3). This value seemed higher than those reported by the literature. However, besides the direct determination of the association constant by Ikemoto [17], which corresponded to a K_m of about 0.25 µM, the most cited K_m were calculated from added $CaCl_2$ by assuming that the apparent binding constant of EGTA for Ca^{2+} was 1.6 μM^{-1} [18], or 3.16 μM^{-1} [19], or 1 μM^{-1} [20]. This range of values was obtained when anionic buffer was used, while as in the medium used for ATPase determination, when cationic buffers were used, the K_{ap} of EGTA for Ca^{2+} was of about 0.1–0.4 μM^{-1} [21]. In our conditions, we have calculated 0.21 μM^{-1} for this constant. Now, if we have based the determination of the free calcium ions on 3.16 μM^{-1} we should have obtained for the $Ca_{0.5}$ a value of about 0.5 µM.

The nature of the calcium dependence was the same for the purified ATPase in R3 as for the native ATPase in R1 (Figs. 2 and 3). Similar results have been reported by other authors [17, 22, 23].

The low Bepridil concentrations of 3.5 and 7 µM raised ATPase activity by 73 and 20%, respectively. This increase was not accompanied by any change in $Ca^{2+}{}_{0.5}$. On the other hand, the inhibition observed for Bepridil concentrations exceeding those quoted above was followed by a slight rise in $Ca^{2+}{}_{0.5}$ (Fig. 2). The only effect of Bepridil on purified ATPase was inhibitory (Figs. 3 and 4).

Given the structure of Bepridil [4], interaction between this compound and carboxylic functions might explain its effect on the calcium-dependent ATPase of sarcoplasmic reticulum.

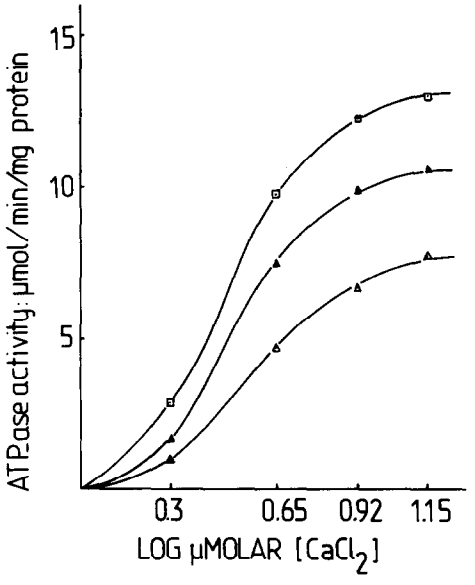

Fig. 3. Effect of Bepridil on the calcium-dependent ATPase activity in the purified R3 fraction. Same conditions as in Fig. 2, except that the amount of proteins in the enzyme suspension was equal to 4 µg.

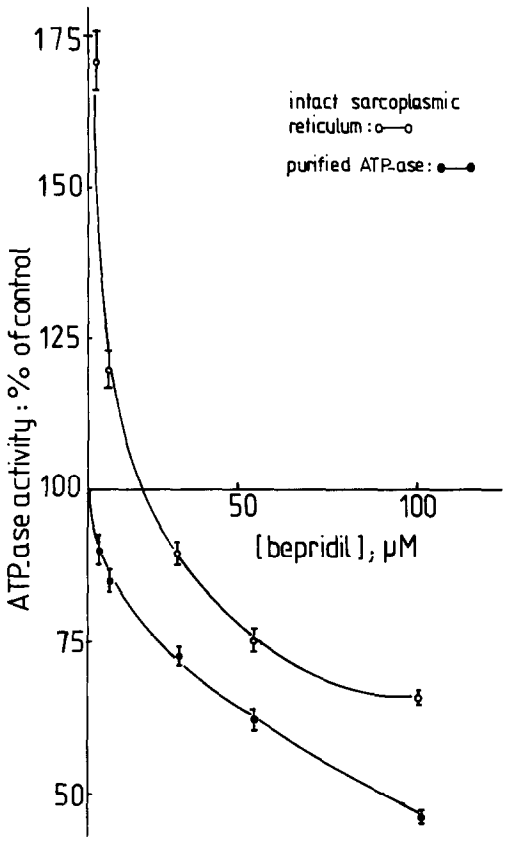

Fig. 4. Biphasic character of the effect of Bepridil on the ATPase activity sarcoplasmic reticulum. Results are expressed as percentages of the control activity obtained without Bepridil. ATPase activity was measured at 14 µM $CaCl_2$.

Table 1 shows that in Hepes–KCl buffer (pH 7.5) Bepridil is insoluble at a concentration of 1 mM. The turbidity of the medium indicates the solubility of the compound. The effects of the various additions led us to think that Bepridil gives rise to soluble complexes by equimolar binding to the carboxylic compounds used. However, a fundamental difference was observed between the behaviour of EDTA and EGTA: the latter only solubilised Bepridil in the presence of equivalent amounts of Ca^{2+}, which was not a necessary condition for solubilisation by EDTA. Note that Mg^{2+} ions were unable to replace Ca^{2+} ions in this respect. The results in Fig. 5 imply that Bepridil causes calcium complexes to dissociate. EDTA formed a complex with 95.3% of the calcium in the medium, whereas EGTA only did this with 83.3% of the calcium. In the presence of Bepridil, these values dropped to 80.7% for EDTA and 60.2% for EGTA.

DISCUSSION

Bepridil forms soluble complexes with calcium ion chelating agents. When these agents are highly water-soluble, as is the case with EDTA, the formation of such complexes does not require the presence of a bivalent cation. On the other hand, when the chelating agent is only slightly water soluble, e.g. EGTA, calcium is indispensable to the formation of soluble complexes with Bepridil, and Mg^{2+} ions cannot replace calcium for this purpose. In all cases, Bepridil causes partial dissociation of calcium complexes.

These results give grounds for thinking that the effect of Bepridil depends on the localisation of the calcium site of the ATPase in sarcoplasmic reticulum, and ATPase activity is indeed controlled by two

Table 1. Bepridil–EDTA and Bepridil–EGTA interactions

Addition	O.D. (540 nm)
2 µmoles Bepridil	1.220
2 µmoles Bepridil + 2 µmoles $CaCl_2$	1.000
2 µmoles Bepridil + 2 µmoles EGTA	1.275
2 µmoles Bepridil + 2 µmoles $CaCl_2$ + 1 µmole EGTA	0.495
2 µmoles Bepridil + 2 µmoles $CaCl_2$ + 2 µmoles EGTA	0.000
2 µmoles Bepridil + 2 µmoles EDTA	0.635
2 µmoles Bepridil + 4 µmoles EDTA	0.000

The turbidity of the medium was measured by light absorption (540 nm). The basic medium contained 2 ml of 2 mM Hepes buffer, pH 7.5, and 130 mM KCl.

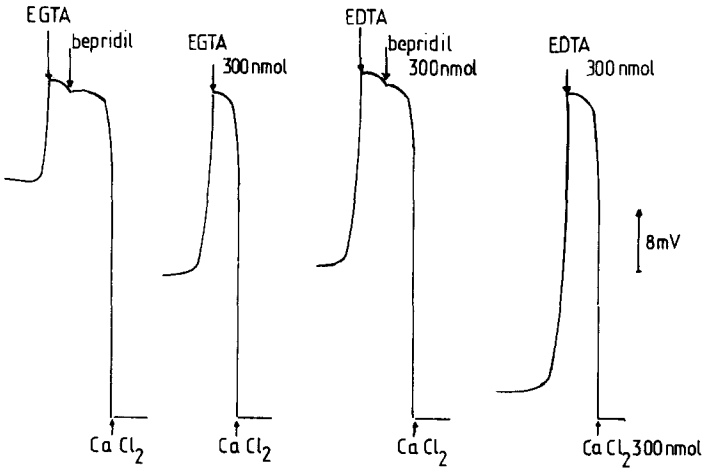

Fig. 5. Effect of Bepridil on the formation of EGTA–Ca^{2+} and EDTA–Ca^{2+} complexes. The free calcium concentration in the medium was determined by a Phillips specific electrode connected to a Phillips ionometer. The potentiometric response was a logarithmic function of the free calcium concentration. The various additions indicated on the figure were made in 5 ml of 3 mM Hepes buffer, pH 7.5, containing 130 mM KCl. The relationship between the response in mV of the electrode and the concentration of Ca^{2+} was given by the following experimental equation: $E = -30 + 28 \log (Ca^{2+})$ where (Ca^{2+}) was expressed as nmoles in the medium.

types of calcium site [17]. Calcium binding to high affinity sites activates the enzyme, whereas binding to low affinity sites inhibits it. High affinity membrane sites face outwards and can therefore be considered as hydrophilic, whereas low affinity sites face inwards [13, 24].

Sarcoplasmic reticulum accumulates Ca^{2+} by hydrolysing ATP. Within the vesicles calcium ions are trapped as insoluble complexes by calsequestrin [24–26]. The increase of Ca^{2+} concentration in the vesicles leads to a decrease in ATPase activity [20, 22]. This decrease may be due to the interaction of internal calcium and the low affinity site of the ATPase. At low concentrations Bepridil, a hydrophobic component, binds only to the membrane causing the dissociation of Ca^{2+} from the low affinity site, hence the increase in the ATPase activity. It is necessary to remind that this situation only occurs at high calcium concentrations (1 mM), which was made possible, in our conditions (Ca^{2+} less than 15 μM), by calcium accumulation inside the vesicle. It is very likely, when purified ATPase was used, that Bepridil only interacts with the high affinity site, since the low affinity site is no more under Ca^{2+} complexes, hence the decrease in the ATPase activity.

It is interesting to note that, during therapeutic use, the plasma concentrations of Bepridil were around 10 μM. These concentrations would be in the range that activated sarcoplasmic reticulum ATPase.

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