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Effects of Carvedilol on Isolated Heart Mitochondria: Evidence for a Protonophoretic Mechanism

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Carvedilol ({1-[carbazolyl-(4)-oxy]-3-[2-methoxyphenoxyethyl)amino]-propanol-(2)}) is a novel compound used in clinical practice for the treatment of congestive heart failure, mild to moderate hypertension, and myocardial infarction. Carvedilol was also shown to protect cardiac mitochondria from oxidative stress events. Because mitochondria are the main suppliers of ATP for cardiac muscle work, a study of the effects of carvedilol in mitochondrial bioenergetics is necessary to fully understand the basis of its protective role in myocardial energetics. In this work we show that carvedilol acts as an uncoupler of oxidative phosphorylation, decreasing mitochondrial electric potential ($\Delta\Psi$) by a weak protonophoretic mechanism. Theoretical studies were carried out to determine the relevance of conformation and proton affinity of the protonable amino side-chain group in the proton-shuttling activity across the inner mitochondrial membrane. BM910228, a hydroxylated metabolite of carvedilol, was also studied for comparison with the parent compound. Implications for the protective role of carvedilol in heart mitochondrial bioenergetics are discussed.

Key Words: **rat heart mitochondria; carvedilol; uncoupler; *ab initio* SCF-MO calculations.**

Carvedilol ({1-[carbazolyl-(4)-oxy]-3-[2-methoxyphenoxyethyl)amino]-propanol-(2)}) (Fig. 1) is a multiple-action neurohormonal antagonist that is clinically used for the treatment of congestive heart failure, mild to moderate hypertension and myocardial infarction (1, 2). Carvedilol competitively blocks β_1, β_2, and α_1 adrenoceptors, having also vasodilating properties that contribute to an overall cardiac sparing effect. Carvedilol has also a potent antioxidant activity, responsible for an increased cardioprotection not shared by other β-blockers (3, 4).

Cardiac function during physiological and pathological situations is closely related to mitochondrial bioenergetics. Mitochondria are the powerhouses of all eukaryotic cells, being source of most of the ATP used for building of body parts. Being a highly energetic-costing process, the work of cardiac muscles requires a constant input of mitochondrial ATP. In several heart malfunctions, mitochondrial bioenergetic is severely afected. The role of mitochondria in ischemic heart disease has already been described (5). Mitochondrial failure has severe consequences for the myocardial cells and may include failure of calcium homeostasis, triggering of apoptotic or necrotic pathways and, of course, suppressed delivery of ATP to heart muscles.

Recent results propose that carvedilol exerts its effects in ischemic heart disease by protecting mitochondrial function. This protection may reach several levels including suppression of oxidative damage to mitochondria (6), which is closely associated to ischemia/reperfusion episodes (7, 8) or inhibition of the cardiac exogenous NADH dehydrogenase (9), an organospecific enzyme located in the inner mitochondrial membrane that has been proposed to be a powerful generator of the superoxide radical (10, 11).

The dissection of the effect of carvedilol in mitochondrial bioenergetics is thus of crucial importance in the study of the mechanisms of its cardioprotection. In our studies (6) we found that carvedilol affects mitochondrial electric membrane potential ($\Delta\Psi$), by depressing it a few millivolts.

In the present paper, the effect of carvedilol in mitochondrial bioenergetics was evaluated in rat heart mitochondria in order to access the probable mechanism of that $\Delta\Psi$ lowering. BM910228 ({1-[3-hydroxy-carbazolyl-(4)-oxy]-3-[(2-methoxyphenoxyethyl)amino]-propanol-(2)}), a hydroxylated analogue of carvedilol (see

Abbreviations used: $\Delta\Psi$, mitochondrial electric potential; BSA, bovine serum albumine; TPP^+, tetraphenylphosphonium cation; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HF, Hartree-Foch; SCF-MO, Self-Consistent Field Molecular Orbital.

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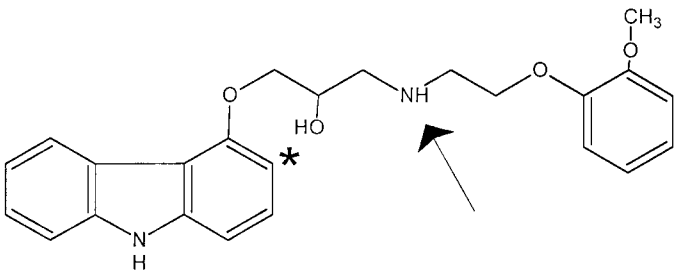

FIG. 1. Structure of carvedilol ({1-[carbazolyl-(4)-oxy]-3-[2-methoxyphenoxyethyl)amino]-propanol-(2)}). The arrow denotes the protonable amino side-chain group (pK_a 7.9); the asterisk indicates the hydroxylated carbon in BM910228.

Fig. 1) was also used in order to establish the mechanism of the $\Delta\Psi$ lowering induced by the parent compound, as it lacks a similar effect in that parameter. Proton affinities of both compounds were also evaluated using *ab initio* quantum mechanical Hartree-Fock Self-Consistent Field Molecular Orbital (HF/SCF-MO) calculations, in order to investigate their acid-base properties and to ascertain the relevance of the hydroxyl group as a substituent in one of the aromatic rings of the molecule.

MATERIALS AND METHODS

Materials. Carvedilol and BM910228 were obtained from Boehringer (Mannheim, Germany) and preparated in dimethyl sulfoxide (DMSO). All other compounds were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of mitochondria from rat heart. Rat heart mitochondria from male Wistar rats (250–300 g) were prepared using a conventional procedure (14), with slight modifications. Briefly, the rats were sacrificed by cervical dislocation and the hearts were immediately excised and minced finely in an ice-cold isolation medium containing 250 mM sucrose, 1 mM EGTA, 10 mM Hepes–KOH (pH 7.4) and 0.1% defatted BSA. The minced blood-free tissue was then resuspended in 40 ml of isolation medium containing 1 mg protease Type VIII (Sigma No. P-5390) per milligram of tissue and homogenized with a tightly fitted homogenizer (Teflon:glass pestle). The suspension was incubated for 1 min (4°C) and then rehomogenized. The homogenate was then centrifuged at 10,000*g* for 10 min (Sorvall RC-5C, Plus, SS 34 rotor, 4°C). The supernatant fluid was decanted and the pellet, essentially devoid of protease, was gently homogenized to its original volume with a loose-fitting homogeneizer. The suspension was centrifuged at 500*g* for 10 min and the resulting supernatant was centrifuged at 10,000*g* for 10 min. The pellet was resuspended using a paint brush and repelleted twice at 10,000*g* for 10 min. EGTA and defatted BSA were omitted from the final washing medium. Mitochondrial protein content was determined by the biuret method calibrated with BSA.

Measurement of mitochondrial transmembrane potential. The mitochondrial transmembrane potential ($\Delta\Psi$) was estimated with a TPP^+ electrode according to Kamo *et al.* (15) without correction for the "passive" binding contribution of TPP^+ to the mitochondrial membranes because the purpose of the experiments was to show relative changes in the potential rather than absolute values. A matrix volume of 1.1 μl/mg protein was assumed. Reactions were carried out, at 25°C, in 2 ml of the standard respiratory medium supplemented with 3 μM TPP^+ and 1 mg of mitochondria. Energized mitochondria were obtained with 8 mM succinate (plus 4 μM rotenone).

Osmotic swelling experiments. The passive proton permeability of the inner mitochondrial membrane was estimated, in both the presence and absence of carvedilol, by means of the swelling of nonrespiring mitochondria in the potassium acetate/valinomycin system (16). Rat heart mitochondria (1 mg) were incubated at 25°C with 2 ml ionic media composed of 135 mM KCH_3COO, 10 mM Hepes, and 0.1 mM EDTA, suplemented with 2 μM rotenone. Valinomycin (1 μg) and FCCP (1 μM) were used in order to increase permeability to potassium and protons, respectively. Decreases of absorvance were measured at 540 nm in a spectrophotometer Jasco V-560. Carvedilol and FCCP (control) were added to the mitochondrial suspension following the adition of valinomycin.

Mitochondrial oxygen consumption. Oxygen consumption of isolated heart mitochondria was monitored polarographically with a Clark oxygen electrode connected to a suitable recorder in a 2-ml thermostated water-jacketed closed chamber with magnetic stirring, at 25°C. The standard respiratory medium consisted of 100 mM KCl, 50 mM sucrose, 10 mM Tris, 30 μM EGTA, 1 mM KH_2PO_4, pH 7.4. Mitochondria were suspended at a concentration of 0.5 mg/ml in the respiratory medium. State IV respiration was measured in the presence of 8 mM succinate (plus 4 μM rotenone).

Ab initio MO calculations. The *ab initio* SCF-MO calculations were performed using the GAUSSIAN 98W program (17), with the split valence basis sets 3-21G (18) (for the total geometry optimizations) and 6-31G* (19) (for the single point energy calculations). Molecular geometries were fully optimized by the Berny algorithm, using redundant internal coordinates (20): the bond lengths to within ca. 0.1 pm and the bond angles to within ca. 0.1°. The final root-mean-square (rms) gradients were always less than 3×10^{-4} hartree $\cdot$ bohr^{-1} or hartree $\cdot$ radian^{-1}.

The proton affinities (PA) were computed by running geometry optimizations plus frequency calculations for the most stable conformations of the molecules—in both the unprotonated and monoprotonated forms—followed by a higher level single point energy calculation at the optimized geometries (refinement of the first, lower level, optimization). The final value, defined as the energy released when a proton is added to the system, is then determined by the energy difference between the unprotonated and protonated molecule of interest, after correction for the corresponding zero-point energies ($PA = E_{corr}(A) - E_{corr}(AH^+)$).

RESULTS

Effects on mitochondrial transmembrane electric potential ($\Delta\Psi$). The effects of carvedilol and BM910228 in the mitochondrial transmembrane electric potential were investigated with a TPP^+ selective electrode. Both carvedilol and BM910228 show a depressing effect in $\Delta\Psi$ of succinate-energized heart mitochondria, with BM910228 being weaker than carvedilol in that action (Fig. 2). BM910228-induced $\Delta\Psi$-depolarizations were approximately less than half the ones provided by carvedilol addition, as seen with a TPP^+-selective electrode. Interestingly, with concentrations as high as 100 μM carvedilol, where an almost maximum of oxygen consumption stimulation was reached, mitochondria were still capable of developing a membrane potential of about -170 mV (without corrections for TPP^+-passive membrane binding) and capable of phosphorylating added ADP, although with a lesser RCR and in a suspressed manner (data not shown).

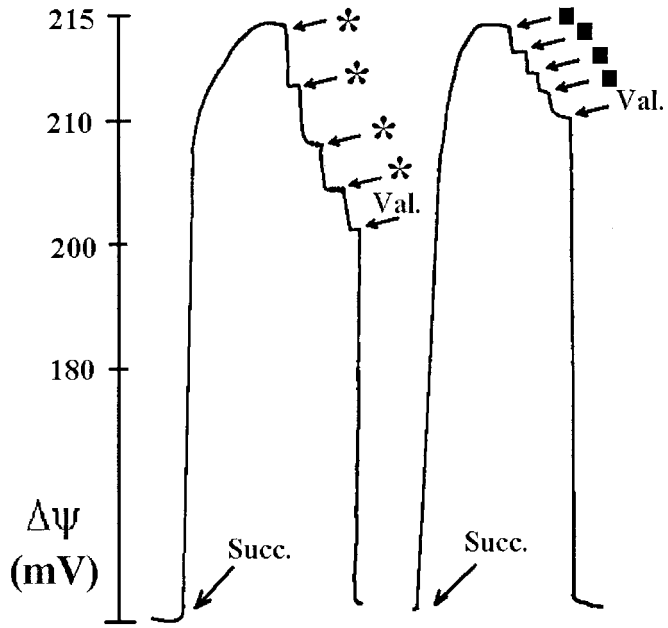

FIG. 2. Typical recordings of mitochondrial $\Delta\Psi$ with a TPP^+-selective electrode. Rat heart mitochondria (0.5 mg/ml) were incubated in 2 ml of the standard respiratory medium supplemented with 3 μM TPP^+. Energization of the mitochondrial population was achieved with 8 mM succinate. 10 μM carvedilol (asterisk) or BM910228 (square) was added where indicated. Valinomycin (1 μg) was added to calibrate potential to baseline. The values of the electric potential were calculated as described under Materials and Methods.

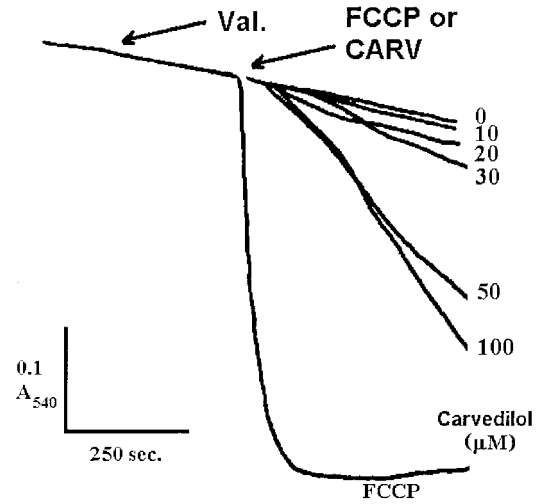

FIG. 3. Representative recording of osmotic swelling of nonenergized rat heart mitochondria (0.5 mg/ml) induced by carvedilol in a potassium acetate media (2 ml) with valinomycin (1 μg). FCCP (1 μM) was added as control. Carvedilol provoked a proton permeabilization in a dose-dependent manner.

Osmotic swelling assays. To see whether the depressing effect in the $\Delta\Psi$ was due to an increased membrane permeability to protons, the swelling of nonrespiring mitochondria was studied with carvedilol in the presence of valinomycin in a potassium acetate media, as described. Acetate crosses the inner mitochondrial membrane in the neutral protonated form. Valinomycin is an ionophore that allows the permeation of K^+. To maintain the electroneutrality of the process, an exit of the protons that come in with acetate should occur. As seen in Fig. 3, carvedilol was able to induce swelling of the mitochondrial population in a dose-dependent manner, indicating a permeabilization to protons of the inner mitochondrial membrane.

Respiration stimulation by carvedilol. Polarographic traces of oxygen consumption were determined in order to establish the effects of carvedilol and BM910228. As seen in Fig. 4, carvedilol stimulates respiration in the absence of exogenous ADP (state IV). When heart mitochondria energized with succinate are challenged with growing concentrations of carvedilol, there is an increase of the oxygen consumption by the mitochondrial preparation. That stimulation is more visible for concentrations higher than 20 μM carvedilol. Carvedilol does not affect oxygen consumption in uncoupled mitochondria (data not shown) and is much weaker than other classic uncouplers, like FCCP, having a maximum of respiration stimulation around 100 μM (for example, under the same conditions, FCCP had a maximum stimulating effect around 1 μM). As seen in Fig. 5, left, carvedilol released oligomycin-inhibited state III respiration, as observed with many uncouplers. That effect is weaker with its metabolite, BM910228 (Fig. 5, right).

Effects of pH media in the depressing effect of carvedilol in $\Delta\Psi$. Two media with different pH (7.4 and 8.4) were used in order to study the relevance of the degree of protonation of the amino group in the lowering of the $\Delta\Psi$. Figure 6 shows the difference in the slopes of the curves relating drug concentration vs $\Delta\Psi$.

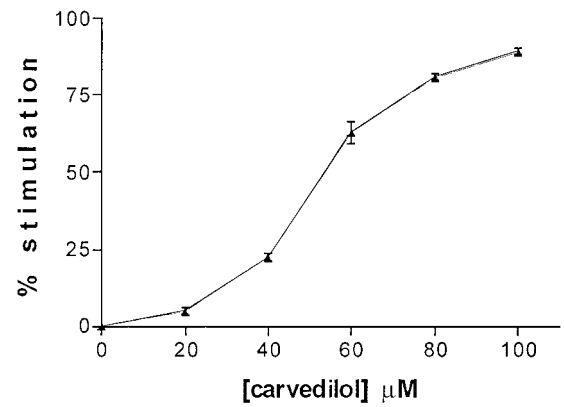

FIG. 4. Stimulation by carvedilol of oxygen consumption in succinate-energized rat heart mitochondria (state IV). Mitochondria (1 mg) were incubated in 2 ml standard respiratory media at 25°C. Results are means ± SEM of three independent experiments.

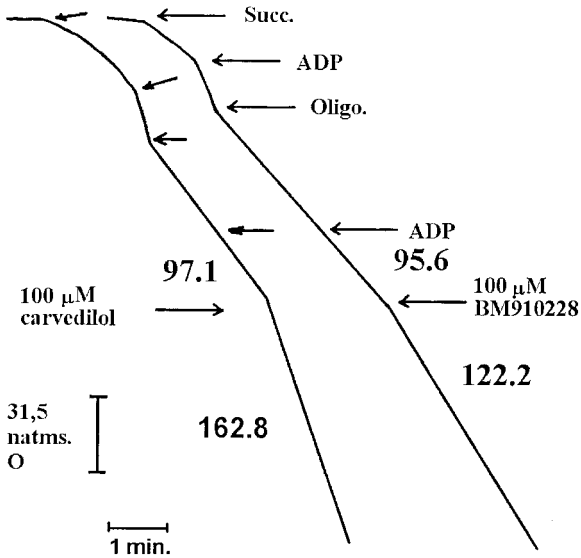

FIG. 5. Comparative effects of carvedilol and BM910228 in oligomycin-restrained respiration in succinate-energized rat heart mitochondria. Mitochondria (1 mg) were incubated in 2 ml standard respiratory media at 25°C. Succinate (8 mM), ADP (50 nmol), and oligomycin (1 μg) were added where pointed. 100 μM of carvedilol (left) or BM910228 (right) was added in order to test their effect in oxygen consumption. Numbers close to curves indicate oxygen consumption in natm O/min/mg protein.

A sharper effect of carvedilol in mitochondrial $\Delta\Psi$ at pH 7.4 was observed, indicating a larger proton shuttling activity.

Ab initio MO calculations. The relationship among the distinct biological effects of carvedilol and BM910228, their conformational preferences and proton affinities was investigated through theoretical methods. Two distinct energy minima were found for the carvedilol molecule, with relative populations (at 25°C) of 71% and 29% ($\Delta E = 2.19$ kJmol^{-1}). For BM910228, in turn, only one stable conformation seems to be significantly populated at room temperature. As to the monoprotonated form of these compounds, one stable geometry was obtained in each case, displaying rather strong energetically favoring O · · · H(O) and O · · · H(N) intramolecular hydrogen bonds (Fig. 7). The proton affinities were calculated to be 980.6 and 997.5 kJmol^{-1} for carvedilol and BM910228, respectively.

DISCUSSION

An analysis of the role that xenobiotics play in mitochondrial bioenergetics is important to understand some of the effects observed in the living organism. Although the direct effects of any particular drug on mitochondria are, most of the times, clear, their precise mechanism is always prone to debate. One clear example is the local anesthetic bupivacaine. Until now, it was generally agreed that bupivacaine exerts an uncoupling effect on oxidative phosphorylation. However, the precise mechanism of that effect has been a point of discussion. Several models were proposed, such as the typical protonophoretic mechanism (13) or the creation of membrane pores (12). Bearing this in mind, the proposal of precise mechanisms for the effects of xenobiotics in mitochondrial bioenergetics is often subject of controversy.

With this work, we intended to study the effects of carvedilol ({1-[carbazolyl-(4)-oxy]-3-[2-methoxyphenoxyethyl)amino]-propanol-(2)}) (Fig. 1), a multiple-action neurohormonal antagonist clinicaly used in the treatment of congestive heart failure, of mild to moderate hypertension, and of myocardial infarction in mitochondrial bioenergetics and tried to glimpse the main mechanism responsible for those effects. To that purpose we studied the effects of carvedilol in mitochondrial $\Delta\Psi$ and oxygen consumption and in the effectiveness to increase proton leak through the inner mitochondrial membrane. The use of BM910228, a hydroxylated metabolite of carvedilol provided some clues to the possible mechanisms of carvedilol, as it did not show similar effects. A great deal of evidence came from studies with the protonable amino side-chain group (pK_a 7.9 (21)), the reactive group supposed to be responsible for the shuttling pathway. Using *ab initio* SCF-MO calculations for both compounds and studying the relevance of the protonation degree of that group, we were able to relate the stability of the protonated form with the ability to work as protonophores.

From Fig. 2, it is clear that carvedilol and BM910228 depress mitochondrial $\Delta\Psi$, albeit in a different manner, as carvedilol provoked more extensive depolarizations. To see whether the depressing effect in the $\Delta\Psi$ was due to an increased membrane permeability to protons, we studied the swelling of nonrespiring mitochondria induced by carvedilol in the presence of valinomycin in a potassium acetate media. As seen on Fig.

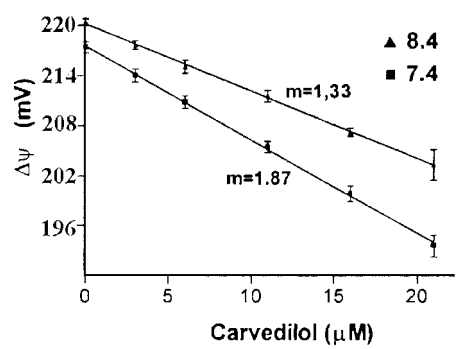

FIG. 6. Dependence of carvedilol $\Delta\Psi$ depressing on media pH. Rat heart mitochondria (1 mg) were incubated with two media with different pHs (7.4 and 8.4) and energized with succinate (8 mM). Carvedilol was added and membrane potentials were determined as described. The slopes of the curves were determined and are shown in the figure. Points are means ± SEM of four independent experiments.

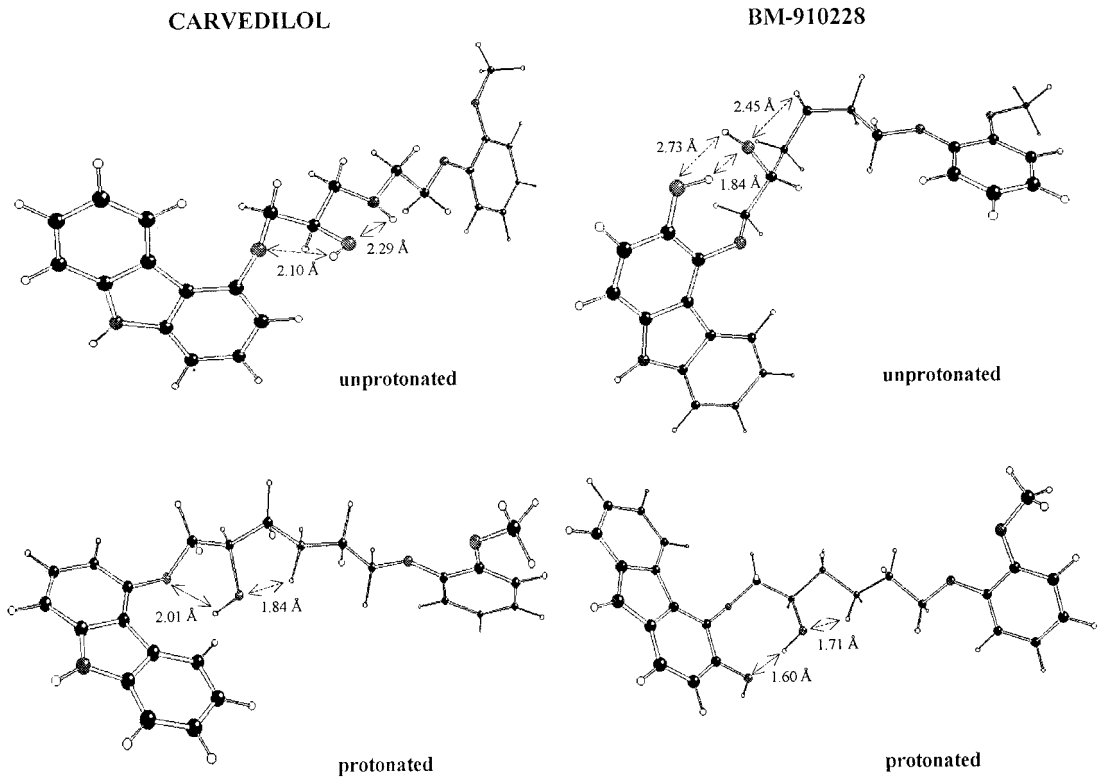

FIG. 7. Most stable conformations for the protonated and unprotonated forms of both carvedilol and BM910228. (Data were collected as described under Materials and Methods.)

3, carvedilol had an FCCP-like effect, in a dose-dependent manner, displaying increased permeabilization to protons. Accordingly, carvedilol stimulated state IV respiration in succinate-energized mitochondria, also in a dose-dependent manner. The stimulatory effect is apparent for concentrations higher than 20 μM (Fig. 4). Carvedilol and BM910228 also circumvented oligomycin-restrained state IV respiration, stimulating oxygen consumption after the addition of oligomycin to succinate energized mitochondria. Once again, carvedilol had a stronger effect (Fig. 5). These results, together with the fact that carvedilol did not seem to inhibit mitochondrial respiratory chain (as uncoupled-respiration in succinate energized mitochondria was not affected by carvedilol in the range of concentrations used), allow us to say that carvedilol showed an uncoupling effect in mitochondria, as it shares many of the features of classic uncouplers (22). Further information would be necessary to establish the precise mechanisms of that uncoupling.

The protonable amino side-chain group has great relevance in compounds that display a classic protonophoretic activity. Taking this into account, we first observed the effects of the degree of protonation in the mitochondrial $\Delta\Psi$ lowering by changing the pH of the reaction media. As seen in Fig. 6, the pH of the reaction media affects the uncoupling activity of carvedilol. With a higher media pH (8.4), carvedilol displayed less efficiency in decreasing $\Delta\Psi$ (slope 1.33) than in a lower pH (7.4) (slope 1.87). That is conceivable if we recall our knowledge of acid-base reactions. At pH 7.4, the ratio between the unprotonated and the protonated forms of carvedilol is 0.32, while at pH 8.4 the ratio is 3.2. In short, this means that a larger number of molecules of carvedilol is protonated at pH 7.4 than at pH 8.4, and so, more protonated molecules are available to shuttle protons through the membrane. In our opinion, that explains the reason for the difference in the slopes of Fig. 6.

Proton affinity studies by *ab initio* SCF-MO calculations showed that carvedilol has a lower proton affinity than BM910228. This weaker affinity for protons (e.g., lower basicity) enables carvedilol to release H^+ more easily within the mitochondrial matrix and thus to act as a more efficient protonophore, although still weak. In turn, BM910228, which displays a higher proton affinity, does not release the carried proton as efficiently as carvedilol in the matrix. The occurrence of intramolecular O · · · H(O) and O · · · H(N) hydrogen type interactions is determinant of the acid-base behavior of these kind of molecules. Thus, the lower proton affinity of carvedilol relative to BM910228 is most probably due to a higher stabilization of the protonated species (containing an NH_2^+ group) in the latter, through an O · · · H(N) interaction (O-H(N) distance = 1.71 Å vs 2.45 Å for the unprotonated molecule), that is

favored by the formation of a neighboring strong, almost colinear O · · · H(O) close contact (O · · · H(O) distance = 1.60 Å, (OHO) angle = 170.5°, Fig. 7). These indications fit in our model for the uncoupling mechanism of carvedilol: we may speculate that it picks up a proton in the cytosolic leaflet of the inner mitochondrial membrane, crossing it in the protonated, positively charged, form, probably driven by the electric potential (negative inside). In the higher pH of the matrix, carvedilol would release the carried proton (more easily than BM910228), crossing back the membrane in the neutral form.

The differential action of carvedilol and BM910228 could also be related to the different lipophilicity of both compounds. In fact, carvedilol is more lipophilic than BM910228 (21), having less difficulty in crossing the inner mitochondrial membrane than his metabolite.

The lowering of mitochondrial $\Delta\Psi$ by carvedilol and, to a lesser extent, by BM910228, may have important biological consequences. For example, the antioxidant activity of carvedilol in mitochondria (16) may be due, in part, to the phenomenon known as "mild uncoupling," in which a small decrease in $\Delta\Psi$ induces a reduction of the reactive oxygen species produced by the mitochondrial respiratory chain (23). The decrease in $\Delta\Psi$ may also be important to avoid calcium overload in pathological events, like in ischemia/reperfusion of the myocardium (24), as the driven force ($\Delta\Psi$) for the entry of calcium is reduced. Both facts may contribute to protect mitochondria during stressful situations.

In conclusion, the present work shows that carvedilol behaves as an uncoupler of mitochondrial respiration, decreasing mitochondrial $\Delta\Psi$ and stimulating respiration by means of a weak protonophoretic mechanism. The diversity of effects diplayed by carvedilol and its metabolite BM910228 are associated to the differences in their molecular conformation. The study of the effect of carvedilol and other cardioprotective compounds in mitochondrial bioenergetics may contribute to a full understanding of their protective properties during pathological events.

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