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Inhibitory effect of carvedilol in the high-conductance state of the mitochondrial permeability transition pore

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Abstract

The mitochondrial permeability transition is a widely studied, but poorly understood, phenomenon in mitochondrial bioenergetics. It has been recognised that this phenomenon is related to the opening of a protein pore in the inner mitochondrial membrane, and that opening of this pore is the cause of some forms of mitochondrial dysfunction. In this work, we propose that carvedilol, a multi-role cardioprotective compound, may act as an inhibitor of the high-conductance state of the mitochondrial permeability transition pore, a conclusion supported by the finding that carvedilol provides differential protection against mitochondrial swelling in sucrose and KCl-based media, and that it is unable to protect against calcium-induced depolarisation of the mitochondrial membrane. We also show that carvedilol inhibits the oxidation of mitochondrial thiol groups and that, beyond causing a slight depression of the membrane potential, it has no inhibitory effect on mitochondrial calcium uptake.

A decrease in the number of oxidised protein thiol groups may be the main mechanism responsible for this selective inhibition of the permeability transition pore in heart mitochondria. These effects may be important for the role of carvedilol in some cardiac pathologies.

1. Introduction

One pillar of the chemiosmotic theory of energy transduction is the impermeability of the inner mitochondrial membrane to solutes that are not transported by natural mitochondrial carriers. This impermeability can be reversed by a phenomenon known as the 'mitochondrial permeability transition' (MPT), caused by the formation of a proteinaceous pore (the mitochondrial permeability transition pore, MPTP), from several mitochondrial proteins (for reviews see Zoratti and Szabò, 1995; Vercesi et al., 1997; Bernardi, 1999). This sudden loss of membrane integrity is perhaps the greatest riddle in mitochondrial studies and is a controversial subject. Agents such as calcium and phosphate (the most studied), pro-oxidants such as *t*-butylhydroperoxide, or thiol cross-linkers such as phenylarsine oxide are inducers of the MTP.

During events such as cardiac ischemia/reperfusion, cardiac mitochondria may suffer severe damage due not only to the increasing payload of oxidative injury but also to the cellular and mitochondrial accumulation of calcium and phosphate (Baker et al., 1988, Kristián and Siesjo, 1996). The role of the mitochondrial permeability transition pore in the reperfusion injury of the heart is well established and may be a determinant of the fate of the cell.

The physiology of the mitochondrial permeability transition pore has been described by some groups. The so-called "low-conductance" state is useful for releasing the calcium accumulated in the mitochondrial matrix and for keeping the mitochondrial transmembrane electric potential ($\Delta\Psi$) within the normal range, avoiding membrane hyperpolarisation (Ichas et al., 1994, 1997; Ichas and Mazat, 1998). The observed $\Delta\Psi$ flickering in single mitochondria is considered to be due to repeated stochastic

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opening and closing of the mitochondrial permeability transition pore (Huser et al., 1998). The transition to a "high-conductance" state depends on the saturation of calcium-binding sites and this state is also responsible for a permanent ATP deficit, leading to cell necrosis (Ichas and Mazat, 1998).

Carvedilol ({1–[carbazolyl–(4)-oxy]-3-[2-methoxyphenoxyethyl)-amino]-propanol-(2)}) is clinically used for the treatment of congestive heart failure, mild to moderate hypertension and myocardial infarction (Dunn et al., 1997). Carvedilol competitively blocks β_1, β_2 and α_1-adrenoceptors, while displaying vasodilating properties. Its antioxidant activity, which is responsible for additional cardioprotective actions not shared by other β-adrenoceptor antagonists (Yue et al., 1992a,b), has also been established.

The effects of carvedilol in ischemic heart disease may be due to the protection of mitochondrial function, for instance, by suppression of oxidative damage to mitochondria (Moreno et al., 1998) or inhibition of the cardiac exogenous NADH dehydrogenase (Oliveira et al., 2000a), an organospecific enzyme located in the inner mitochondrial membrane that has been proposed to be a powerful generator of the superoxide radical (Nohl, 1987a,b). Carvedilol also displays a weak protonophoretic activity (Oliveira et al., 2000b).

The aim of this study was to investigate the effects of carvedilol in the mitochondrial permeability transition induced by calcium/phosphate in rat heart mitochondria. To this purpose, we followed several events associated with the opening of the mitochondrial permeability transition pore.

2. Materials and methods

2.1. Materials

Carvedilol and BM910228 were obtained from Boehringer (Mannheim, Germany) and prepared in dimethylsulfoxide (DMSO). All other compounds were purchased from Sigma (St. Louis, MO)

2.2. Animals

Male Wistar rats (250–300 g), housed at 22 + 2°C under artificial light for 12-h light/day cycle and with access to water and food ad libitum, were used throughout the experiments. The research reported here was carried out in accordance with the National Requirement for Vertebrate Animal Research.

2.3. Isolation of mitochondria from rat heart

Rat heart mitochondria from male Wistar rats (250–300 g) were prepared as previously described (Oliveira et al., 2000a). Mitochondrial protein content was determined by the biuret method, calibrated with bovine serum albumin.

2.4. Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential ($\Delta\Psi$) was estimated with a TPP^+ electrode according to Kamo et al. (1979) 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 media (200 mM sucrose, 10 mM Tris–Mops, 10 μM EGTA, 5 mM KH_2PO_4) supplemented with 3 μM TPP^+ and 1 mg of mitochondria. Energised mitochondria were obtained with 8 mM succinate (plus 4 μM rotenone and 0.5 μg oligomycin). Carvedilol was pre-incubated with the mitochondrial suspension for 3 min before mitochondrial energisation.

2.5. Determination of mitochondrial swelling

Changes in mitochondrial volume were followed by monitoring the classic decrease in absorbance at 540 nm with a Jasco V-560 spectrophotometer. The assays were performed in 2 ml of standard media with 4 μM rotenone, 0.5 μg oligomycin and 8 mM succinate and 1 mg protein. In a separate set of experiments, 200 mM sucrose was replaced by 100 mM KCl or by a combination of either 100 mM polyethyleneglycol 200 or 1000 plus 60 mM KCl. Carvedilol was pre-incubated with the mitochondrial preparation for 3 min at 25°C. In some experiments, simultaneous measurements of mitochondrial $\Delta\Psi$ and swelling were obtained using a Spectronic 20 spectrophotometer and a TPP^+-selective electrode immersed in 4 ml of the standard media and with 2 mg of mitochondrial protein.

2.6. Determination of mitochondria protein thiol content

To determine the antioxidant protective effect of carvedilol on thiol groups in sucrose and KCl-based media, we used a variation of Ellman's method (Riddles, 1979). After each incubation, two 0.75-ml samples were taken and added to 0.75 ml of sulphosalicilic acid 4% each and submitted to centrifugation at 15 500 g for 15 min in a Sigma 3K30 Centrifuge at 4°C.

The supernatant was discarded and the pellet was suspended in 1.5 ml of phosphate buffer 100 mM, pH 8. The suspension was sonicated and a 1.0-ml sample was diluted to 2.6 ml in phosphate buffer medium containing 385 μM dithionitrobenzoic acid. The absorption was measured at 412 nm.

2.7. Calcium accumulation by heart mitochondria

The Ca^{2+} storage capacity of mitochondria was estimated spectrophotometrically at 660 nm in a Jasco 560 spectrophotometer, as described (Solem and Wallace, 1993). Mitochondria were suspended at 0.25 mg/ml in 2 ml of medium added to a cuvette maintained at 25°C. After 1 min, Ca^{2+} was added (200–300 nmol), and mitochondria were energised with 10 mM succinate.

3. Results

The effect of increasing concentrations of carvedilol on Ca^{2+}/P_i-induced mitochondrial swelling was studied by measuring the changes in the suspension absorbance at 540 nm in a sucrose-based media. Carvedilol protected heart mitochondria from the mitochondrial permeability transition in a dose-dependent manner (Fig. 1). Fig. 2 shows that the addition of carvedilol to the mitochondrial suspension affected the magnitude of swelling in a time-dependent manner. Carvedilol, per se, neither stopped nor reversed the mitochondrial permeability transition but needed at least 3 min to have an inhibitory effect. To see whether this protection extended to the usually observed membrane depolarisation, we measured the variation in the mitochondrial electric potential ($\Delta\Psi$) after the addition of calcium pulses. A representative recording is shown in Fig. 3, where it can be seen that carvedilol did not have any protective effect against calcium-induced depolarisation, whatever the concentration. Simultaneous recording of mitochondrial $\Delta\Psi$ and swelling showed that although there was an inhibitory effect on mitochondrial swelling, no difference was observed in the $\Delta\Psi$ (data not shown).

This led us to propose that carvedilol caused a selective inhibition of the "high-conductance" state of the mitochondrial permeability transition pore. Sucrose, but not proton, influx would be prevented by carvedilol, with consequent membrane depolarisation.

This hypothesis was further corroborated by the measurement of mitochondrial swelling in a KCl-based media.

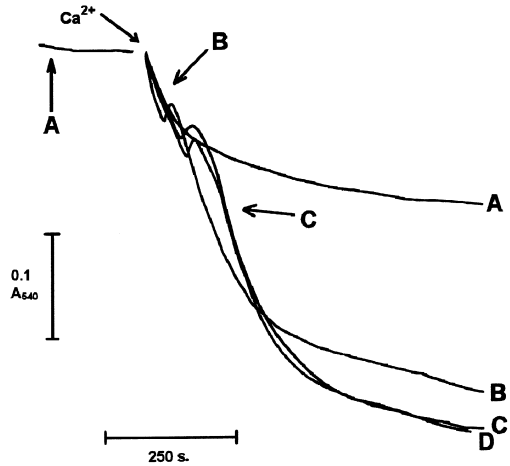

Fig. 2. Effect of the time-lapse after carvedilol addition. Mitochondria were incubated as described in the legend of Fig. 1. Carvedilol (20 μM) was added at different times (A, B and C). The letters at the ends of curves correspond to the times of addition. (D) No additions. The recording is representative.

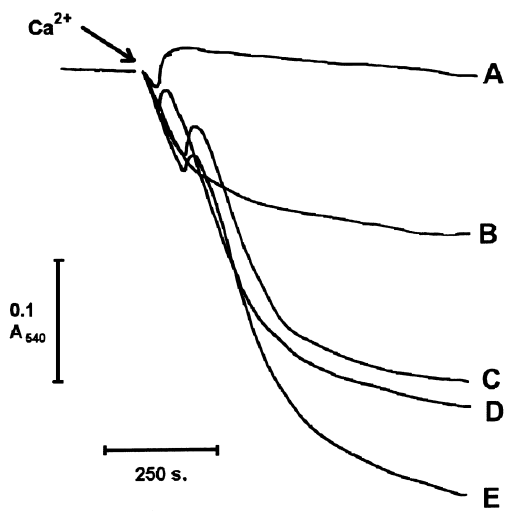

Fig. 1. Typical recording of mitochondrial swelling (decrease of absorbance at 540 nm) induced by calcium (500 μM). Heart mitochondria (0.5 mg/ml) were incubated in 2 ml of media composed of 200 mM sucrose, 10 mM Tris–Mops, 10 μM EGTA and 5 mM KH_2PO_4, supplemented with 4 μM rotenone, 0.5 μg oligomycin and 8 mM succinate. After a basal line was established, calcium was added. Where referred, carvedilol was pre-incubated with mitochondria for 3 min at 25°C. The additions were as follows: (A) 1 μM cyclosporin A, (B) 20 μM carvedilol, (C) 10 μM carvedilol, (D) 5 μM carvedilol and (E) no additions.

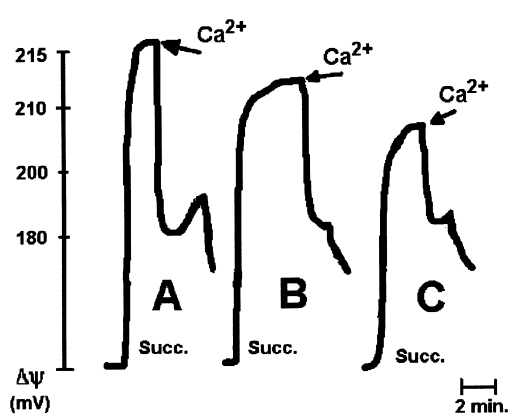

Fig. 3. Mitochondrial electric potential measurements obtained with a TPP^+-selective electrode. Calcium (500 μM) was added in order to induce the mitochondrial permeability transition pore, under the same conditions as described in the legend of Fig. 1 (media supplemented with 3 μM TPP^+). Carvedilol (10 and 20 μM) was pre-incubated for 3 min with heart mitochondria. Individual calibrations with valinomycin were made for each of the experiments to set the baseline. The recordings are representative of five different preparations.

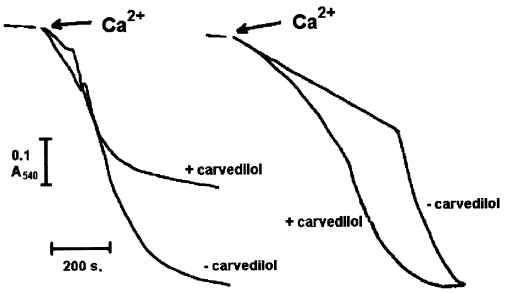

Fig. 4. Different levels of protection afforded by 20 μM carvedilol against mitochondrial swelling (decrease of absorbance at 540 nm) induced by 600 μM calcium in a sucrose (200 mM sucrose, 10 mM Tris–Mops, 10 μM EGTA and 5 mM KH_2PO_4, plus 4 μM rotenone, 0.5 μg oligomycin and 8 mM succinate, left recording) or KCl-based (100 mM KCl, 10 mM Tris–Mops, 10 μM EGTA and 5 mM KH_2PO_4, supplemented with 4 μM rotenone, 0.5 μg oligomycin and 8 mM succinate, right recording) medium. Carvedilol was pre-incubated with mitochondria for 3 min.

We found out that 20 μM carvedilol had no effect on the magnitude of swelling (Fig. 4, right), which contrasts sharply with the effect of the same concentration of carvedilol in a sucrose-based media, where an inhibition is clearly visible (Fig. 4, left). Carvedilol even increased the rate of swelling in KCl-based media. This would mean that the entry of the larger molecule, sucrose, was restricted, in contrast to the entry of much smaller ions. In other words, carvedilol caused a selective exclusion of solutes. When media based on polyethyleneglycols with molecular weights 200 and 1000 were used, and despite an overall inhibitory effect against mitochondrial swelling, we noticed that the inhibition was more pronounced in the $polyethyleneglycol_{1000}$-based media (data not shown).

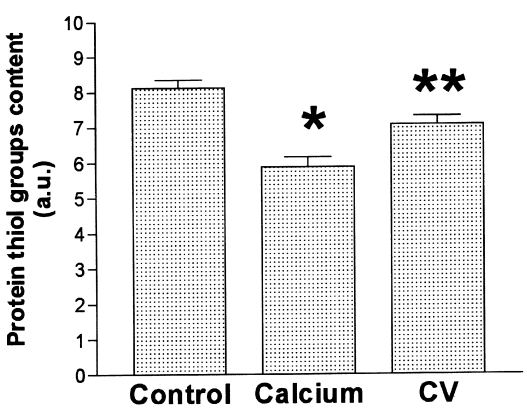

Fig. 5. Oxidation of mitochondrial protein thiol groups determined as described in Section 2. Mitochondria were incubated with calcium (500 μM) for 3 min before the assays (Ca and CV). When present, carvedilol (CV) was pre-incubated with mitochondria for 30 min before calcium addition. Media were the same as described in the legend of Fig. 1. Results are means ± S.E.M. of four different preparations. $^{*}p < 0.001$, compared with control, $^{**}p < 0.05$, compared with calcium without carvedilol.

The oxidation of protein thiol groups is known to be one of the hallmarks of induction of the mitochondrial permeability transition. We determined the extent of carvedilol-induced protection against protein thiol group oxidation. We found out that carvedilol decreased the extent of protein thiol group oxidation induced by Ca^{2+}/P_i. Fig. 5 shows the extent of protection in the sucrose-based media. The results in KCl-based media were very similar (data not shown).

Measurements of calcium movements, using Arsenazo as a calcium sensitive probe, showed that carvedilol had a dual action on mitochondrial calcium accumulation: a negligible effect when calcium concentrations were below the threshold for pore opening (i.e., calcium uptake was normal) (Fig. 6, down) and a protective effect when the calcium concentration was enough to cause pore activation (Fig. 6, up). Thus, carvedilol increased the rate and extent of calcium accumulation at high calcium concentrations, demonstrating a protective effect, while not having any effect on calcium uptake at low calcium concentrations. These results showed that the decrease in mitochondrial $\Delta\Psi$ was not enough to decrease calcium entry.

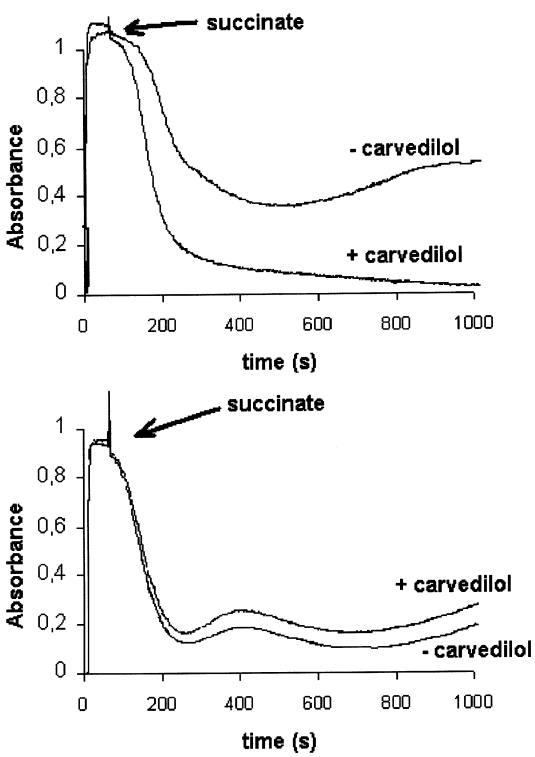

Fig. 6. Determination of extra-mitochondrial calcium movements using Arsenazo as a calcium-sensitive probe. Two millilitres of assay medium (200 mM sucrose, 10 μM EGTA–Tris, 5 mM KH_2PO_4, 10 mM Tris, pH 7.4) was supplemented just before the Ca^{2+} capacity assays with 100 μM of Arsenazo III, 2 μM rotenone and 1 μg/ml oligomycin. Mitochondria were suspended at 0.25 mg/ml. After 1 min of incubation, Ca^{2+} was added (300 nmol—upper recording, 200 nmol—lower recording), and mitochondria were energised with 10 mM succinate. Carvedilol 10 μM was used with a 3-min previous incubation. Recordings are representative.

4. Discussion

In this work, we studied the effect of carvedilol on the permeability transition in heart mitochondria, a deleterious event often associated with mitochondrial and cellular dysfunction.

As cardiac cells handle high concentrations of calcium, heart mitochondria are much more resistant to calcium than liver mitochondria, for example. In our work, it was necessary to use 5 mM P_i and 500 μM calcium or higher to induce mitochondrial swelling and depolarisation. In liver mitochondria, the calcium concentrations used to induce mitochondrial permeability transition pore opening are usually five times lower. As cardiomyocytes need a constant supply of mitochondrial ATP to ensure their functions, their survival is threatened when mitochondrial dysfunction occurs. In a condition of ischemia/reperfusion, where there is increased oxidative stress, calcium and P_i, the mitochondrial permeability transition occurs (Crompton and Costi, 1990; Halestrap et al., 1998; Baker et al., 1988; Kristián and Siesjo, 1996). The use of drugs to protect cardiac cells from the mitochondrial permeability transition is most valuable in these situations.

In this work, we showed that carvedilol is an inhibitor of the mitochondrial permeability transition, exerting a dose-dependent effect against mitochondrial swelling (Fig. 1). The specific inhibitor of the mitochondrial permeability transition pore, the immunosuppressive peptide cyclosporin A, completely abolished mitochondrial swelling, as expected (Broekemeier et al., 1989). The protective effect of carvedilol was time-dependent (Fig. 2) because it took up to 3 min to attain the maximum inhibitory effect. This lag time may correspond to the insertion of carvedilol into the mitochondrial membrane because carvedilol has a great tendency to insert into membranes (Cheng et al., 1996). The opening of the mitochondrial permeability transition pore occurred simultaneously with mitochondrial depolarisation (drop in $\Delta\Psi$). No protective effect of carvedilol against Ca^{2+}-induced depolarisation was observed when a TPP^+-selective electrode was used. As seen in Fig. 3, carvedilol was unable to maintain $\Delta\Psi$ after a strong Ca^{2+} pulse. The only visible effect was a slight decrease in $\Delta\Psi$, concomitant with a weak protonophoretic activity (Oliveira et al., 2000b). This was confirmed by results obtained with simultaneous recordings of mitochondrial $\Delta\Psi$ and swelling, where inhibition was noticed in the swelling component but not in the $\Delta\Psi$ depolarisation component.

Carvedilol was unable to inhibit mitochondrial swelling in a KCl-based media (Fig. 4) but showed a stronger inhibitory effect in a polyethyleneglycol$_{1000}$-based media than in a polyethyleneglicol$_{200}$-based media. This may mean that carvedilol only inhibited one of the substrates of the mitochondrial permeability transition pore, the high-conductance state, which is responsible for mitochondrial swelling in sucrose-based media. In other words, carvedilol probably inhibited the entry of large molecules into the mitochondrial matrix but had no effect on the entry of smaller molecules. In our opinion, this is the reason why we observed different degrees of protection induced by carvedilol according to the size of the external osmotically active molecules.

Carvedilol also inhibited the oxidation of mitochondrial protein thiol groups (Fig. 5), an event associated with opening of the mitochondrial permeability transition pore. As proposed by Zago et al. (2000), the redox state of endogenous pyridine nucleotides and hence the redox state of mitochondrial thiols can determine the solute selectivity of the permeability transition pore. This may explain the different degrees of protection provided by carvedilol in sucrose and in KCl-based media, in spite of the observed similarity in inhibition of protein thiol group oxidation. The decrease in thiol group oxidation can be explained by the antioxidant activity of carvedilol (Yue et al., 1992a,b; Moreno et al., 1998). This agrees with results obtained with BM910228 ({(1-[3-hydroxy-carbazolyl-(4)-oxy]-3-[(2-methoxyphenoxyethyl)-amino]-propanol-(2)}), a hydroxylated metabolite of carvedilol, that has greater antioxidant activity (Yue et al., 1994), which inhibited mitochondrial swelling in the sucrose-media to a greater extent than did carvedilol in the same concentrations (data not shown).

Protection against thiol group oxidation may be the main mechanism by which carvedilol inhibits the mitochondrial permeability transition pore in its high conductance state. We also considered that carvedilol, due to its protonophoretic properties, might depress calcium influx into the mitochondria, thus inhibiting the permeability transition in an indirect form. Our results with the Arsenazo technique ruled out this hypothesis. Carvedilol not only did not have an inhibitory effect on calcium influx at low calcium loads (Fig. 6, down) but it also inhibited mitochondrial calcium release induced by high calcium loads (Fig. 6, up).

On the basis of our results, we propose that carvedilol may act as a selective inhibitor of the high-conductance state of the mitochondrial permeability transition pore, as described previously (Ichas et al., 1994, 1997; Ichas and Mazat, 1998), or, as proposed in some models (Petronilli et al., 1999), as an inhibitor of the long lasting pore opening responsible for the observed extensive swelling in the sucrose-based media.

Since the low-conductance state of the mitochondrial permeability transition pore has been associated with protective mechanisms against calcium overload, oxidative stress and $\Delta\Psi$ hyperpolarisation (Ichas and Mazat, 1998; Huser and Blatter, 1999), it is possible that the cardioprotective action of carvedilol is, partly, due to a selective inhibition of a deleterious high-conductance state of the mitochondrial permeability transition pore, without inhibiting the low-conductance state of the pore, which is considered to have physiological functions. Work is underway in our laboratory to determine the precise mechanism of carvedilol actions and to identify the components of the

mitochondrial permeability transition event. This is very important in order to explain some results that appeared in the literature concerning non-conventional, mitochondrial permeability transitions (Sokolove and Haley, 1996; Kushnareva and Sokolove, 2000). In the future, we are also interested in finding out whether carvedilol actually binds to a mitochondrial protein that forms the pore.

In conclusion, we propose that carvedilol is an inhibitor of the high-conductance state of the mitochondrial permeability transition pore, acting on mitochondrial swelling and calcium efflux without preventing $\Delta\Psi$ depolarisation or swelling in a ionic media. With regard to the clinical relevance of the finding, although carvedilol is not able to prevent mitochondrial $\Delta\Psi$ depolarisation, it might prevent structural damage to the mitochondria. The antioxidant activity of carvedilol may help prevent the irreversible mitochondrial dysfunction caused by the permeability transition.

Acknowledgements

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