0026-895X/11/7905-814–822$25.00
MOLECULAR PHARMACOLOGY
Vol. 79, No. 5
70342/3679750
Mol Pharmacol 79:814–822, 2011
Printed in U.S.A.

A Common Mechanism Links Differently Acting Complex II Inhibitors to Cardioprotection: Modulation of Mitochondrial Reactive Oxygen Species Production[S]

Stefan Dröse, Lea Bleier, and Ulrich Brandt

Molecular Bioenergetics Group, Cluster of Excellence Frankfurt "Macromolecular Complexes," Medical School, Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany

Received December 4, 2010; accepted January 28, 2011

ABSTRACT

In this study, we have analyzed the effect of different cardioprotective complex II inhibitors on the mitochondrial production of reactive oxygen species (ROS) because ROS seem to be essential for signaling during preconditioning to prevent ischemia/reperfusion injury. Despite different binding sites and concentrations required for half-maximal inhibition—ranging from nanomolar for the Q site inhibitor atpenin A5 to millimolar for the succinate analog malonate—all inhibitors modulated ROS production in the same ambivalent fashion: they promoted the generation of superoxide at the Q_o site of complex III under conditions of "oxidant-induced reduction" but attenuated ROS generated at complex I due to reverse electron transfer. All inhibitors showed these ambivalent effects independent of the presence of K^+. These findings suggest a direct modulation of mitochondrial ROS generation during cardioprotection via complex II inhibition and question the recently proposed role of complex II as a regulatory component of the putative mitochondrial K_{ATP} channel.

Introduction

Increased mitochondrial production of reactive oxygen species (ROS) is usually linked to deleterious consequences involved in several pathophysiological settings, including ischemia/reperfusion injury (Halestrap et al., 2007). However, the consequences of increased ROS during hypoxic periodes are complex and apparently ambivalent (Hanley and Daut, 2005; Halestrap et al., 2007): whereas ROS are unambiguously involved in damaging cardiac tissue upon reperfusion, transient ROS generation seems to be essential for signaling during ischemic and pharmacological preconditioning, cardioprotective mechanisms that attenuate ischemia/reperfusion injury. There are indications that ROS produced at the Q_o site of mitochondrial complex III are involved in ischemic and pharmacological preconditioning (Vanden Hoek et al., 1998; Oldenburg et al., 2003), but the underlying molecular mechanism is as unclear as the nature of the generators of deleterious ROS during reperfusion. We presented a straightforward rationale to mechanistically explain the ambivalent effects of the cardioprotective K_{ATP} channel opener diazoxide on mitochondrial ROS generation (Dröse et al., 2009). On the one hand, diazoxide that also inhibits mitochondrial complex II (Schäfer et al., 1971; Hanley et al., 2002; Dröse et al., 2006) directly attenuates ROS generation by succinate fueled rat heart mitochondria due to reverse electron transfer (RET) from complex II into complex I. On the other hand, diazoxide stimulates superoxide production at the Q_o site of complex III under conditions of "oxidant-induced reduction," (i.e., in the presence of the Q_i site inhibitor antimycin A) (Dröse et al., 2009). This stimulation that has been confirmed in an independent study (Liu et al., 2010) can be explained by increased oxidation of the Q-pool after complex II inhibition. We have shown that ROS generation at Q_o site of complex III is maximal if the Q-pool is partially reduced implying that superoxide is generated by reverse electron transfer from reduced heme b_L to molecular oxygen with ubiquinone serving as a redox mediator (Dröse and Brandt, 2008). These findings were supported by investigations of the Osyczka group (Borek et al., 2008; Sarewicz et al., 2010). Our proposed mechanism contradicts the view that a semiquinone intermediate formed during normal turnover at the Q_o

This work was supported by the Deutsche Forschungsgemeinschaft [Grant SFB 815 Redox-Regulation, Project A2].

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.110.070342.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: ROS, reactive oxygen species; DQA, SAN 549, 2-*n*-decyl-quinazolin-4-yl-amine; FCCP, carbonyl-cyanide-*p*-trifluoro-methoxy-phenylhydrazone; HRP, horseradish peroxidase; mitoK_{ATP}, mitochondrial ATP-sensitive K^+; Q-pool, ubiquinone pool; RET, reverse electron transfer; RHM, rat heart mitochondria; SMP, submitochondrial particle; TTFA, 2-thenoyltrifluoroacetone.

site of complex III is the electron donor for superoxide (Muller et al., 2002; Cape et al., 2009).

Another controversially discussed issue concerns the question of whether diazoxide exerts its cardioprotective effects solely via complex II inhibition and ROS generation (Hanley et al., 2002; Dröse et al., 2006, 2009), or if a putative mitochondrial K_{ATP} channel is involved as an independent effector (Liu et al., 2010), an upstream regulator (Oldenburg et al., 2003), or downstream target (Facundo et al., 2007; Pasdois et al., 2008) of ischemic preconditioning- and pharmacological preconditioning-induced ROS. It has been proposed that complex II may be a component or regulator of the mitoK_{ATP} channel (Ardehali et al., 2004; Wojtovich and Brookes, 2009). It was shown that the highly potent and specific inhibitor atpenin A5 (Miyadera et al., 2003) that binds to the ubiquinone binding site of complex II (Horsefield et al., 2006) had the same cardioprotective effects in cardiomyocytes and Langendorff-perfused rat hearts as ischemic preconditioning, diazoxide, and malonate (Wojtovich and Brookes, 2009). The effect of atpenin A5 was independent of complex II inhibition, and the authors suggested that opening of the putative mitoK_{ATP} channel is the underlying mechanism. An alternative explanation was given by Jaggar and coworkers (Adebiyi et al., 2008), who analyzed the effect of different K_{ATP} channel openers on the vasodilation of mouse myogenic mesenteric arteries. These authors came to the conclusion that diazoxide and atpenin A5 induce vasodilation not via K_{ATP} channel opening but by inhibiting complex II leading to ROS-dependent K_{Ca} and voltage-gated K^+ (K_v) channel activation. In the present study, we have analyzed the effects of four inhibitors [atpenin A5, diazoxide, malonate, 2-thenoyltrifluoroacetone (TTFA)] that have different binding sites within complex II and that exert inhibitory and cardioprotective effects (Wojtovich and Brookes, 2009) over a wide range of concentrations. Atpenin A5 and TTFA bind to the Q-site and malonate to the dicarboxylate binding site of complex II (Fig. 1), whereas the binding site of diazoxide is not known. All substances only affected mitochondrial ROS generation when succinate was the predominant substrate. The Q site inhibitors atpenin A5 and TTFA showed the same ambivalent K^+-independent effects as reported previously for diazoxide and malonate (Dröse et al., 2009), and this was clearly correlated to complex II inhibition. Our findings suggest that inhibition of complex II directly modulates the mitochondrial ROS generation during cardioprotection via lowering the membrane potential ($\Delta\Psi$) and oxidation of the ubiquinone pool, respectively.

Materials and Methods

Materials. 2-*n*-Decyl-quinazolin-4-yl-amine (DQA, SAN 549) was obtained from AgrEvo (Frankfurt, Germany), Amplex Red (*N*-acetyl-3,7-dihydrophenoxazine) was purchased from Invitrogen (Carlsbad, CA) and atpenin A5 was from Alexis Biochemicals (Lausen, Switzerland). Fatty acid-free bovine serum albumin was from SERVA (Heidelberg, Germany). Superoxide dismutase (from bovine liver), horseradish peroxidase (HRP), TTFA, diazoxide (7-chloro-3-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide), stigmatellin, and all other chemicals were from Sigma-Aldrich (St. Louis, MO). Diazoxide, atpenin A5, antimycin A, DQA, stigmatellin, TTFA, carbonyl-cyanide-*p*-trifluoro-methoxy-phenylhydrazone (FCCP), oligomycin, and Amplex Red were dissolved in dimethyl sulfoxide. Because diluted atpenin A5 solutions had a limited stability, they were freshly prepared from a 1 mM stock before starting the experiments.

Preparation of Submitochondrial Particles. Submitochondrial particles from bovine heart mitochondria were prepared as described previously (Dröse et al., 2006). The preparations had a protein concentration of 33.6 mg/ml (27.4 μM heme *b* and 71.6 μM heme aa_3) and 18.2 mg/ml (32.3 μM heme *b* and 35.3 μM heme aa_3), respectively.

Isolation of Intact Rat Heart Mitochondria. Rat heart mitochondria (RHM) were isolated at 4°C as reported previously (Dröse et al., 2006). Diced ventricular tissue was minced and washed with a solution containing 300 mM sucrose, 10 mM Na^+/HEPES, pH 7.2, and 0.2 mM EDTA. The tissue was treated with trypsin (~0.1 mg/ml) for 15 min and homogenized twice before adding soybean trypsin inhibitor (~0.3 mg/ml). The heart mitochondria were subsequently washed, centrifuged, and resuspended in solution containing 300 mM sucrose, 10 mM Na^+/HEPES, pH 7.4, 0.2 mM EDTA, and 1 mg/ml fatty acid-free bovine serum albumin. All RHM preparations had respiratory control ratios (ADP-stimulated state 3/state 4 with malate/glutamate) of 10 or greater.

Detection of Reactive Oxygen Species. ROS were detected by the Amplex Red/HRP assay in fluorescence mode. The assay was calibrated with known H_2O_2 concentrations (0–5 μM). Resorufin formation was measured at 30°C in a SpectraMax M2^e multimode reader (Molecular Devices, Sunnyvale, CA) at the following settings: sensitivity low; top read; excitation, 540 nm; emission, 600 nm (fixed bandwidth, 9 nm for excitation/emission); and cutoff filter, 590 nm. Typically, 24 μg of submitochondrial particles (SMPs) were used in a total volume of 200 μl of reaction mixture, containing 50 μM Amplex Red, 0.1 U/ml HRP, 400 U/ml superoxide dismutase, 75 mM sodium phosphate, pH 7.4, 1 mM EDTA, and 1 mM $MgCl_2$. The respiratory chain was fueled by 0.25 mM NADH or 5 mM succinate, respectively. RHM (12–33 μg of protein/well depending on the preparation) was diluted in a reaction mixture containing 50 μM Amplex Red, 0.1 U/ml HRP, 200 mM sucrose, 10 mM Tris/HCl, 10 mM potassium or sodium phosphate, pH 7.0, 10 mM $MgSO_4$, 100 μM ATP (if not indicated otherwise), and 2 mM EDTA. In contrast to our previous study (Dröse et al., 2009), bovine serum albumin was omitted in the

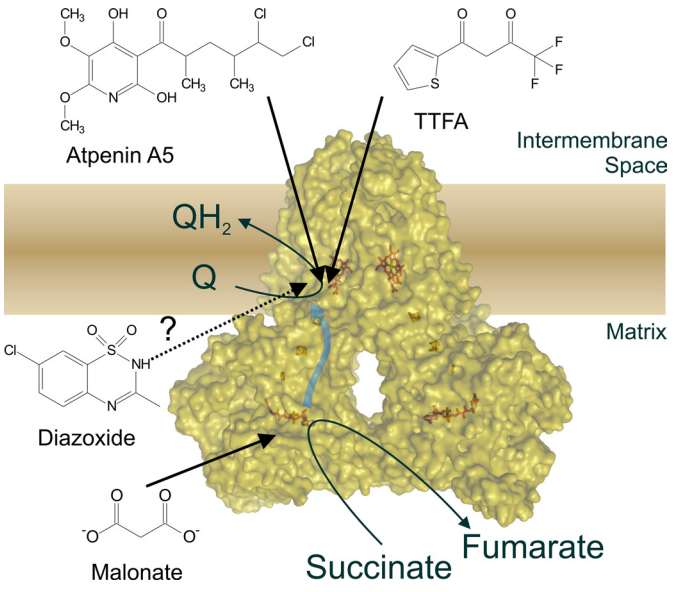

Fig. 1. Structures and binding sites of inhibitors within complex II. Although atpenin A5 and TTFA bind to the Q_p site at the interface between subunits B (iron-sulfur protein), C (CybL), and D (CybS) (Miyadera et al., 2003; Sun et al., 2005), the succinate analog malonate binds to the substrate (succinate) binding site located in subunit A (flavoprotein). Kinetic data indicate that the K_{ATP} channel opener diazoxide inhibits complex II with respect to succinate in a noncompetitive manner (Schäfer et al., 1971), but the exact binding site is not known.

buffer, because variable binding of hydrophobic inhibitors was observed. RHM were fueled either by malate/glutamate (4.8/5.6 mM) or 5 mM succinate, respectively. Inhibitors/effectors were added in the following final concentrations (if not indicated otherwise): 1 μM antimycin A, 1 μM stigmatellin, 1 μM DQA, 50 nM FCCP, and 2 μg/ml oligomycin. As we have described previously and discussed in more detail (Dröse et al., 2009), the ROS generation of intact and highly coupled rat heart mitochondria strongly depends on the quality of the preparation and the time elapsed between completion of the preparation protocol and the actual start of the ROS measurement. Therefore, it was necessary to keep time variations as small as possible. To meet these requirements, we performed the Amplex Red/HRP assay in 96-well plates and restricted the number of inhibitor concentrations under different conditions to 5. Within each preparation, all of the measurements with one type of inhibitor were done at the same time, usually in combination with a second inhibitor (pairs were atpenin A5/TTFA and diazoxide/malonate). The maximal time between two sets of 20-min measurements was 15 min; thus, all data were obtained within 1 h after completion of the respective RHM preparation. However, the immediate application of the RHM inevitably resulted in some variation in the protein amount per assay, which might be in part responsible for the relatively high standard deviations under some conditions (see below), especially for the high-affinity inhibitor atpenin A5.

Measurement of Mitochondrial Respiration. The rate of mitochondrial respiration was monitored at 25°C using an Oxygraph-2k system (OROBOROS Instruments GmbH, Innsbruck, Austria), equipped with two chambers and DatLab software version 4.2. Rat heart mitochondria (0.12–0.33 mg of protein) were added to 2 ml of a buffer containing 200 mM sucrose, 10 mM potassium or sodium phosphate, pH 7.0, 10 mM Tris/HCl, 10 mM $MgSO_4$, and 2 mM EDTA. The mitochondria were fueled by 4.8/5.6 mM malate/glutamate. The respiratory control factor was determined as ratio between state 3 and state 4 of respiration after the addition of 2 mM ADP. For the determination of succinate-oxidase inhibition, 100 μM ATP and 5 mM succinate (in the absence of complex I inhibitors) were added, and the measurements were performed at the same time as the related Amplex Red/HRP assays.

Succinate Oxidase Activity. Submitochondrial particles (240 μg of protein) were added to a 2 ml reaction mixture containing 75 mM sodium-phosphate, pH 7.4, 1 mM $MgCl_2$, 1 mM EDTA, and 5 mM sodium succinate. Note that the SMP concentration was identical with that in the Amplex Red/HRP assay, because especially for the high-affinity complex II inhibitor atpenin A5, some influence of the protein concentration on the resulting I_{50} value was observed (data not shown). Oxygen consumption was measured at 25°C using the Oxygraph-2k system (OROBOROS Instruments).

Results

Effect of Atpenin A5 and TTFA on the ROS Production by Submitochondrial Particles. We have shown previously that complex II inhibitors like malonate, oxaloacetate, or diazoxide can increase the production of superoxide in SMPs at the Q_o site of complex III if 1) succinate is the predominant substrate of the respiratory chain, and 2) if complex III is in the state of "oxidant-induced reduction" (i.e., if reoxidation of cytochrome *b* is blocked at the Q_i site, e.g., by the inhibitor antimycin A) (Dröse and Brandt, 2008; Dröse et al., 2009). Although oxaloacetate and malonate inhibit complex II competitively to the substrate succinate, diazoxide acts in a noncompetitive manner (Schäfer et al., 1971), but its exact binding site is not known. In a first set of experiments we analyzed whether the inhibitors atpenin A5 and TTFA that bind to the Q-site of complex II (Miyadera et al., 2003; Horsefield et al., 2006) affect ROS production of bovine SMP (Fig. 2). SMPs have the advantage that involvement of a putative mitochondrial K_{ATP} channel can be excluded, that electrons can be fed directly into the different respiratory chain complexes, and that ROS production can be detected quantitatively with the Amplex Red/HRP assay without any interference of the mitochondrial ROS defense system. Although TTFA did increase the background fluorescence of the Amplex Red/HRP assay, neither the kinetic rate nor the resolution of the assay was affected by the addition of TTFA per se (results not shown). Both inhibitors did not markedly affect the ROS production when electrons were supplied via complex I (by NADH) or complex III (by *n*-decyl-ubiquinol; results not shown) as had been observed previously with oxaloacetate, malonate, or diazoxide (Dröse and Brandt, 2008; Dröse et al., 2009). When electrons were supplied via complex II by the addition of succinate, the inhibitors alone did not show any effect either (Fig. 2), indicating that complex II inhibition per se did not lead to an increase in ROS

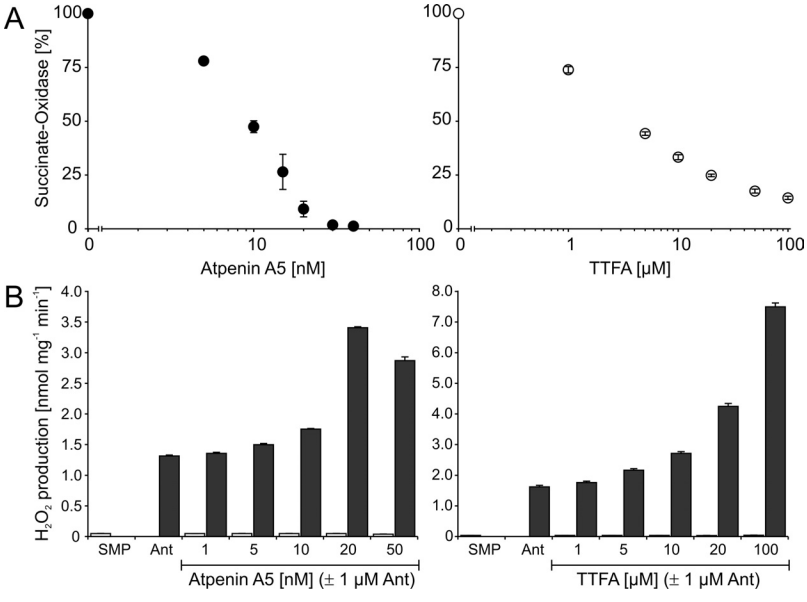

Fig. 2. Effect of the Q site inhibitors atpenin A5 and TTFA on ROS production of SMP. A, effect of atpenin A5 and TTFA on the succinate oxidase activity of SMP. The O_2 flux at 25°C was 60.0 ± 2.3 nmol · mg^{-1} · min^{-1} and 70.4 ± 0.7 nmol · mg^{-1} · min^{-1}, respectively. Error bars indicate ± S.D. (n = 2). Note that the protein concentration in the assay was identical with that in the Amplex Red/HRP assay. B, effect of different inhibitor concentrations on the H_2O_2 generation of succinate-fueled SMP in the absence (open columns) and presence (dark gray columns) of 1 μM antimycin A (Ant); n = 3, data given ± S.D.

production under the conditions applied. However, under conditions of oxidant-induced reduction (i.e., in the presence of antimycin A), atpenin A5 and TTFA considerably stimulated ROS generation at the Q_o site of complex III (Fig. 2B). This increase was completely sensitive to the Q_o site inhibitor stigmatellin (data not shown) and correlated well with the degree of succinate oxidase inhibition. A marked increase occurred when succinate oxidase was inhibited more than 75%. The highest rates were detected when 80 to 90% of the activity was inhibited at 100 μM TTFA or 20 nM atpenin A5. At near complete inhibition, the rates decreased again (i.e., with 50 nM atpenin A5). This is consistent with the model that the redox state of the Q-pool controls superoxide production of the Q_o site of complex III that is maximal with a partially oxidized Q-pool as induced by complex II inhibition under these conditions (Dröse and Brandt, 2008). Furthermore, these results are in accordance with results obtained with oxaloacetate, malonate, and diazoxide (Dröse and Brandt, 2008; Dröse et al., 2009) indicating that just the degree of complex II inhibition and the resulting oxidation of the Q-pool controls ROS production by complex III but not the binding site or the mechanism of inhibition.

Effect of Different Complex II Inhibitors on ROS Production by Coupled Rat Heart Mitochondria. Only highly coupled RHM preparations were used in our investigation (respiratory control factors with malate/glutamate as substrates were at least 10). All measurements were performed in a buffer containing 100 μM ATP but omitting the complex V inhibitor oligomycin that is usually included in studies dealing with the putative mitoK_{ATP} channel, because this represents more physiological conditions (Riess et al., 2008) and bypasses complications due to the induction of a high rate of RET-induced ROS production when succinate is the predominant substrate (Dröse et al., 2009). As observed previously with diazoxide and malonate (Dröse et al., 2006, 2009), TTFA and atpenin A5 had no effect on mitochondrial ROS production when the respiratory chain was fueled via complex I by malate and glutamate (results not shown). In a

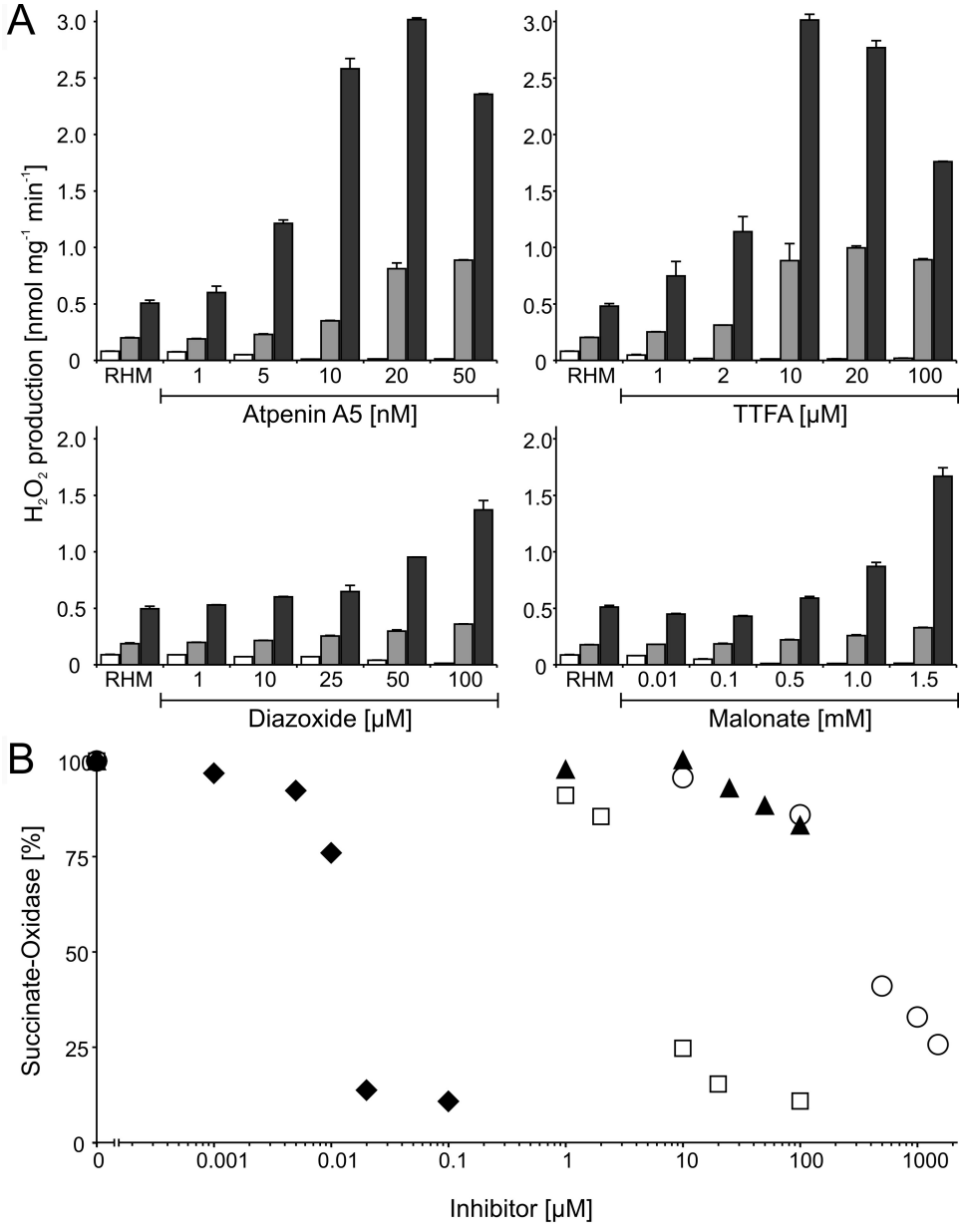

Fig. 3. Effect of different complex II inhibitors on ROS generation of intact rat heart mitochondria in the presence of 5 mM succinate. A, measurements with one representative RHM preparation (17 μg of protein/well) are shown. The H_2O_2 generation in a buffer of 200 mM sucrose, 10 mM Tris/HCl, pH 7.0, 10 mM potassium phosphate, 10 mM $MgSO_4$, 100 μM ATP, and 2 mM EDTA was detected by the Amplex Red/HRP assay. The RHM-independent background (resorufin generation in the absence of RHM) was substracted. Measurements were performed with succinate alone (open columns), + 1 μM antimycin A (light gray columns), + 1 μM antimycin A and 50 nM FCCP (dark gray columns) and the indicated concentrations of complex II inhibitors; $n = 2$, data given ± S.D. B, the effects of the complex II inhibitors (♦, atpenin A5; □, TTFA; ○, malonate; ▲, diazoxide) on the succinate-oxidase activity of this RHM preparation was monitored in the identical buffer by an Oxygraph-2k system. The respiratory rate (JO_2) was 5.8 ± 0.4 nmol · mg^{-1} · min^{-1} ($n = 4$).

potassium-containing buffer, all four tested complex II inhibitors had ambivalent effects on mitochondrial H_2O_2 production when succinate was the substrate (Fig. 3A and 4). Figure 3 shows a representative set of data obtained with a single RHM preparation to reveal differences in the rates of ROS production. To illustrate the effect of batch-dependent variations, Fig. 4 summarizes the changes relative to the respective basal rate with three different biological samples. If succinate was applied as the sole electron donor for the respiratory chain, an H_2O_2 production rate of approximately 100 pmol $\cdot$ mg^{-1} $\cdot$ min^{-1} (variation 44–234 pmol $\cdot$ mg^{-1} $\cdot$ min^{-1}, $n = 6$) was detected. This can be attributed completely to ROS generation by complex I due to reverse electron transfer from complex II, because it was sensitive to the complex I inhibitor DQA and to uncoupling by 50 nM FCCP (Supplemental Fig. 1). In contrast, the addition of the complex V inhibitor oligomycin increased the rate 5- to 7-fold (Supplemental Fig. 1). This indicated that the high membrane potential required as the driving force for RET-induced ROS production (Votyakova and Reynolds, 2001) was already reduced by the addition of ATP that was probably partly hydrolyzed to ADP. All complex II inhibitors attenuated ROS production (Fig. 3A and 4). A comparison with the inhibition of succinate oxidase activity revealed that an inhibition by only 25% was sufficient to almost completely impede complex I-related ROS production under these conditions. If the respiratory chain was inhibited by a Q_i site inhibitor of complex III (antimycin A), ROS production switched from complex I (due to the reduction of $\Delta\Psi$) to the Q_o site of complex III (Dröse et al., 2009). A reduction in complex II activity now caused an increase in mitochondrial ROS production at the Q_o site of complex III (Fig. 3 and 4). This can be explained by an increased oxidation of the Q-pool due to complex II inhibition that favors superoxide production at the Q_o site of complex III from reduced heme b_L (Dröse and Brandt, 2008). The data for atpenin A5, TTFA, and diazoxide indicated that there was a clear correlation of complex II inhibition and stimulation of the antimycin A-induced ROS production under these conditions. Already at moderate inhibition of complex II, a slight increase of the rate could be observed. The highest rates were detected when succinate oxidase was inhibited by more than 50% by TTFA and atpenin A5 and a stimulation of the antimycin A induced ROS-production up to ~1 nmol $\cdot$ mg^{-1} $\cdot$ min^{-1} (range, 0.6–1.4 nmol $\cdot$ mg^{-1} $\cdot$ min^{-1}, $n = 6$) was observed. In contrast, diazoxide exerted only ~20% inhibition at the highest applied concentration of 100 μM (Fig. 4), resulting in a moderate increase of the ROS production. A somewhat different relation was observed for malonate: whereas the maximal concentration of 1.5 mM inhibited succinate oxidase by 75%, the antimycin A-induced ROS production was only stimulated to the level of diazoxide. This difference may be due to the requirement of a membrane potential or ΔpH for the carrier-mediated uptake of the dicarboxylate acid malonate into the mitochondrial matrix that was present in the respiratory measurements with succinate (Fig. 3B) but impeded in the ROS measurements by the addition of antimycin A (Fig. 3A). In SMPs, the same malonate concentrations stimulated the antimycin A-induced ROS production to a much higher degree and much more effectively than diazoxide (Dröse and Brandt, 2008; Dröse et al., 2009; data not shown). In all cases, the addition of the uncoupler FCCP dramatically increased the antimycin A-induced ROS generation at the Q_o site of complex III, and in combination with "optimal" concentrations of TTFA or atpenin A5 rates of ~3 nmol $\cdot$ mg^{-1} $\cdot$ min^{-1} were usually achieved (range, 2.7–5.4 nmol $\cdot$ mg^{-1} $\cdot$ min^{-1}, $n = 6$). Especially under these conditions, it was obvious that further inhibition of complex II and hence further oxidation of the Q-pool resulted in a reduction of the H_2O_2 production, giving the complex II inhibitor profiles a bell-shaped appearance. The emerging picture from these measurements is that all tested complex II inhibitors had ambivalent effects on ROS production of intact RHM respiring on succinate and that their specific characteristics were directly related to their potency of complex II inhibition.

The Modulatory Effect of Atpenin A5 and TTFA on ROS Production Is Not Dependent on Potassium. Because it has been proposed that complex II is a component or regulator of the proposed mitoK$_{ATP}$ channel (Ardehali et al., 2004; Wojtovich and Brookes, 2009), we tested whether the

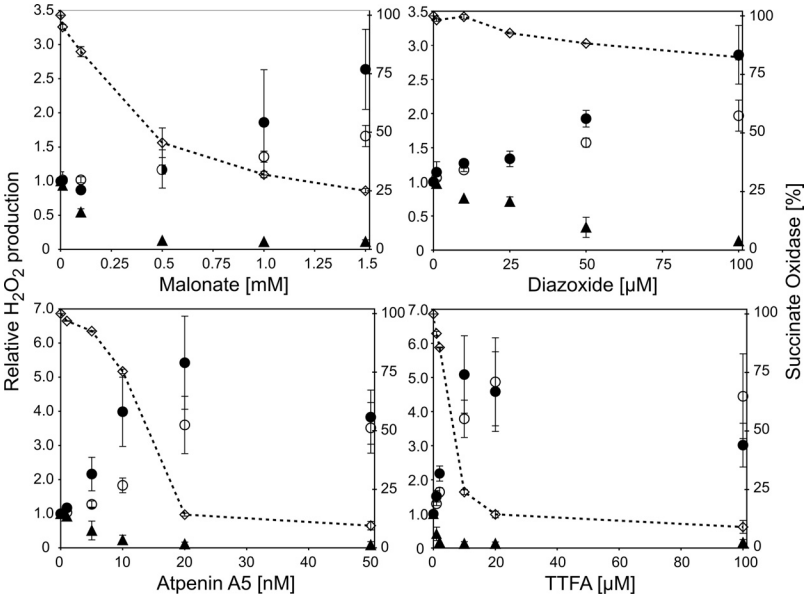

Fig. 4. Relative effect of different complex II inhibitors on the ROS generation of intact rat heart mitochondria in presence of 5 mM succinate. This figure includes a replot of the data presented in Fig. 3. To compensate preparation-dependent differences, the respective ground rate of each data set was set to one and the relative effects of different complex II inhibitors were calculated. Succinate alone (▲), + 1 μM antimycin A (○), and + 1 μM antimycin A and 50 nM FCCP (●). Mean values for three different preparations ± S.D. are shown; each of the duplicates per assay was treated independently (hence $n = 6$). Inhibition of succinate-oxidase: ◇, respiratory rate (JO_2) was 6.1 ± 0.4 nmol $\cdot$ mg^{-1} $\cdot$ min^{-1} ($n = 4$ for each inhibitor, overall $n = 12$).

absence or presence of potassium ions had any influence on the observed modulatory effects of the two Q-site inhibitors atpenin A5 and TTFA. If there was a functional association between the putative mitoK$_{ATP}$ channel and complex II, we would have expected an increase in ROS production only with potassium-containing buffer, because then depolarization of the membrane potential by opening of a K^+ channel should increase the ROS production at the Q_o site of complex III, as was seen with the uncoupler FCCP (Dröse et al., 2009). Furthermore, the atpenin A5-dependent attenuation of the ROS production at complex I due to RET should only occur with potassium-containing buffer if the putative mitoK$_{ATP}$ channel was involved. However, overall, the effects of atpenin A5 and TTFA on ROS generation and inhibition of state 4 respiration were quite similar in potassium- and sodium-containing buffers (Figs. 5 and 6). There was even a general trend that the H_2O_2 production rates in sodium-containing buffer were slightly higher than in potassium-containing buffer. In addition, respiratory rates and coupling rates of the RHM were largely unaffected (results not shown). In most preparations, a shift toward somewhat lower complex II inhibitor concentrations for maximal ROS production in the sodium-containing buffer was observed. However, this was not always obvious, because other factors like the actual protein concentration in the assay affected the inhibitor-ROS production ratio, especially in case of the tight binding inhibitor atpenin A5. These data clearly indicated that potassium ions did not affect the ability of atpenin A5 and TTFA to modulate mitochondrial ROS production in an ambivalent way when succinate was the predominant substrate. Hence, our data indicate that the inhibitors induced substrate-dependent stimulation or attenuation of the superoxide production by direct inhibition of complex II.

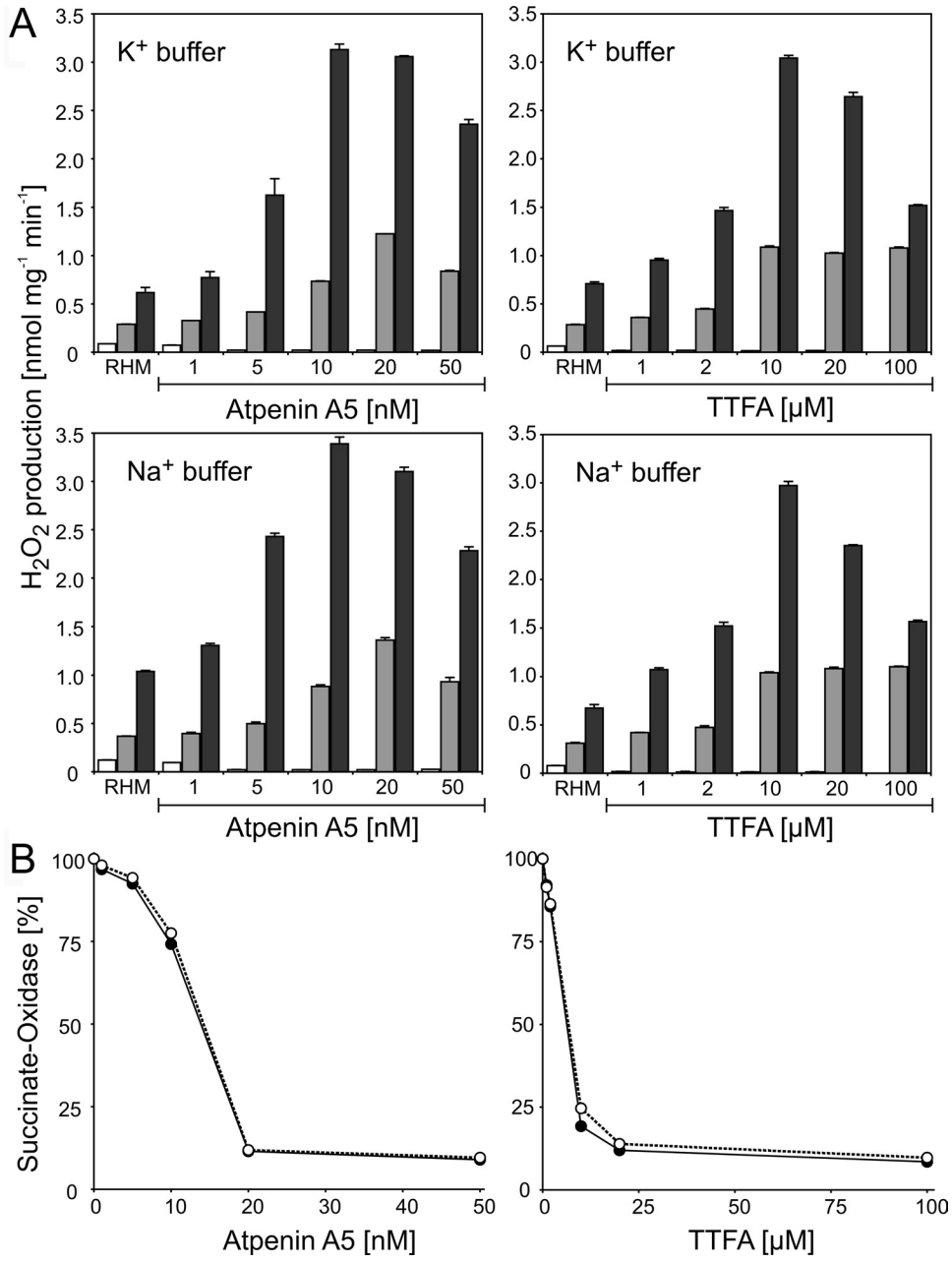

Fig. 5. Effect of atpenin A5 and TTFA on the ROS generation of intact rat heart mitochondria in presence of 5 mM succinate. A, measurements with one representative RHM preparation (12 μg of protein/well) are shown. The H_2O_2 generation in a buffer of 200 mM sucrose, 10 mM Tris/HCl, pH 7.0, 10 mM potassium phosphate (K^+ buffer, top), or 10 mM sodium phosphate (Na^+ buffer, middle), 10 mM $MgSO_4$, 100 μM ATP, and 2 mM EDTA was detected by the Amplex Red/HRP assay. General conditions were as described in legend to Fig. 3. B, the effects of the complex II inhibitors on the succinate-oxidase activity of the respective RHM preparation was monitored by an Oxygraph-2k system: ●, K^+ buffer; ○, Na^+ buffer. The respiratory rates were 3.5 ± 1.4 nmol · mg^{-1} · min^{-1} for the K^+ and 3.6 ± 1.4 nmol · mg^{-1} · min^{-1} for the Na^+ buffer (n = 2 for each buffer).

Discussion

Our results demonstrate that all complex II inhibitors, despite their different binding sites (Fig. 1) and a wide range of affinities, affected ROS production by the respiratory chain in the same way. All compounds exhibited comparable ambivalent effects when succinate was the predominant substrate: they attenuated ROS production at the level of complex I by reverse electron transfer, whereas they stimulated ROS generation at the Q_o site of complex III under conditions of oxidant-induced reduction. In this respect, complex II activity modulates these two respiratory chain complexes (Dröse et al., 2009) that are generally regarded as the main ROS producers within mitochondria (Kowaltowski et al., 2009; Brand, 2010). A common principle exerting these effects seems evident, because we observed a strict correlation between the degree of inhibition and the modulating effects on ROS production for all compounds. Three of the investigated complex II inhibitors have been shown to be cardioprotective in concentrations (Burwell et al., 2009) that are in good agreement with those affecting ROS production of RHM. Because ROS play a central role in preconditioning and in cardiac damage (Halestrap and Pasdois, 2009), a link between mitochondrial ROS modulation and cardioprotection seems plausible. Furthermore, our data question the proposed role of complex II in the composition or regulation of the putative mitochondrial K_{ATP} channel (Ardehali et al., 2004; Wojtovich and Brookes, 2009), because for none of the inhibitors tested, including atpenin A5, did we observe an effect of K^+ ions on the ambivalent modulation of ROS production.

If succinate is the main substrate of mitochondria, ROS are predominantly produced at complex I due to reverse electron transfer against the gradient of redox potentials from complex II via ubiquinone to complex I, which requires a high membrane potential as the driving force (Votyakova and Reynolds, 2001). According to our model (Dröse et al., 2009), this is the predominant mode of ROS production upon reperfusion after succinate accumulation during ischemia. Although it had been claimed that such high succinate concentrations necessary for RET do not occur under physiological conditions, Starkov (2008) has shown that succinate accumulates in mitochondria under hypoxic conditions. As can be seen from our results, already a moderate inhibition of complex II by ~25% can minimize ROS production due to RET, probably by reducing $\Delta\Psi$ and the supply of electrons. This is in agreement with the observation that already a small decrease of $\Delta\Psi$ by only 5% can reduce the ROS generation by 95% (Votyakova and Reynolds, 2001). Compared with the other complex II inhibitors, diazoxide seems to be more potent, minimizing complex I ROS generation even at a lower degree of complex II inhibition. Although this could in principle be explained by the (additional) opening of a mitoK_{ATP} channel, which would also reduce $\Delta\Psi$, our previous studies have shown that the effects of diazoxide are not potassium-dependent (Dröse et al., 2009). It seems more likely that the weak uncoupling properties of diazoxide have to be considered here (Holmuhamedov et al., 2004; Dröse et al., 2006; Kopustinskiene et al., 2010), because uncoupling attenuates ROS generation by RET.

Because complex I releases superoxide completely into the mitochondrial matrix (St-Pierre et al., 2002), reducing its ROS production by complex II inhibitors or uncouplers would largely attenuate production of deleterious ROS. On the matrix site, ROS can not only damage mitochondrial DNA, they are also a main determinant for the opening of the mitochondrial permeability transition pore, which seems to be involved in reperfusion injury (Halestrap and Pasdois, 2009). The X-ray structural analysis of mitochondrial complex I (Hunte et al., 2010) indicates that the ubiquinone reduction site is located ~25 Å above the membrane plane in the peripheral arm. Thus, even if mitochondrial complex I generates superoxide not exclusively at the tightly bound flavin mononucleotide (Galkin and Brandt, 2005; Kussmaul and Hirst, 2006), but also at the ubiquinone binding site (Lambert and Brand, 2004; Brand, 2010), superoxide would have to be released into the matrix from this site.

Although complex II inhibitors reduce complex I-derived ROS of succinate-fueled mitochondria, the same inhibitors can dramatically increase superoxide production at the Q_o site of complex III under conditions of oxidant-induced reduc-

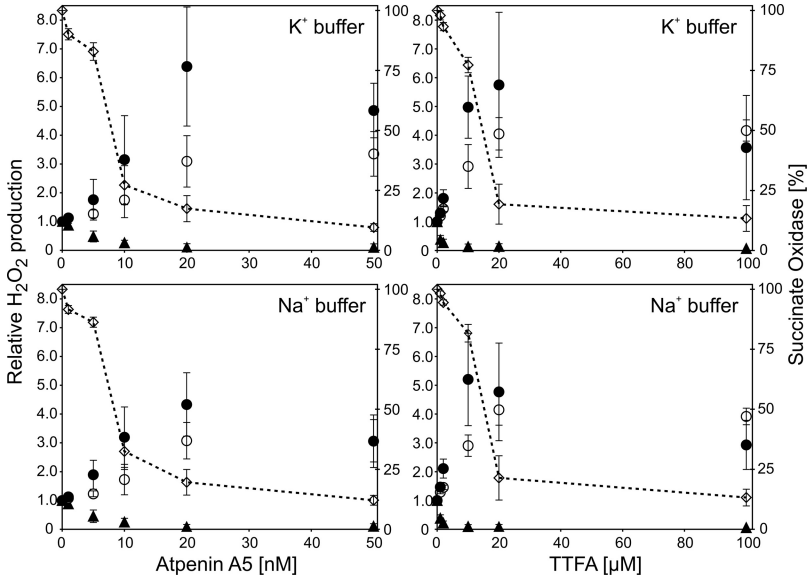

Fig. 6. Relative effect of atpenin A5 and TTFA on the ROS generation of intact rat heart mitochondria in K^+ and Na^+ buffer. RHM were fueled by 5 mM succinate. This figure includes the data presented in Fig. 5. To compensate preparation-dependent differences, the respective ground rate of each data set was set to 1, and the relative effects of different complex II inhibitors were calculated. Succinate alone (▲), + 1 μM antimycin A (○), and + 1 μM antimycin A and 50 nM FCCP (●). Mean values for three different preparations ± S.D. are shown; each of the duplicates per assay was treated independently (hence $n = 6$). Inhibition of succinate-oxidase: ◇, respiratory rates (JO_2) were 5.9 ± 2.1 nmol · mg^{-1} · min^{-1} for the K^+ and 5.7 ± 1.8 nmol · mg^{-1} · min^{-1} for the Na^+ buffer ($n = 6$ for each condition).

tion. Inhibition of complex II under these conditions results in an increased oxidation of the Q-pool, which favors superoxide production at the Q_o site of complex III by reverse electron transfer from reduced heme b_L (Borek et al., 2008; Dröse and Brandt, 2008; Sarewicz et al., 2010). These results are in line with our proposal that complex II might regulate the generation of signaling ROS at complex III during the preconditioning phase (Dröse et al., 2009). Experimental data indicate that ROS produced at the Q_o site of complex III are involved in ischemic and pharmacological preconditioning (Vanden Hoek et al., 1998; Oldenburg et al., 2003). This is also in good agreement with the fact that ROS produced at the Q_o site are predominantly released into the intermembrane space (St-Pierre et al., 2002; Muller et al., 2004), where they could turn on protective cellular pathways without damaging the mitochondrial DNA or inducing mitochondrial permeability transition pore opening (Dröse et al., 2009).

Several published data support the notion that the ambivalent effects of complex II inhibitors observed in our in vitro systems also occur under physiological conditions (i.e., in cardiomyocyte cultures or in Langendorff-perfused rat hearts). It was shown in two initial studies that generation of diazoxide and the hypoxia-induced ROS in the preconditioning phase was attenuated by the Q_o site inhibitor myxothiazol (Vanden Hoek et al., 1998; Oldenburg et al., 2003). Furthermore, Dos Santos and colleagues (Pasdois et al., 2008) observed a transient increase of ROS generation in perfused hearts during preconditioning with diazoxide, although it also decreased ROS produced during reperfusion. It has also been shown that diazoxide and malonate can attenuate ROS generation occurring after and during a longer ischemic period and upon reperfusion in isolated rat heart mitochondria (Ozcan et al., 2002). It remains an open question of whether the observed modulating effects of complex II inhibitors on mitochondrial ROS production are exclusively responsible for the cardioprotective action or whether a mitochondrial K_{ATP} channel is involved or is even a determining factor. This is controversially discussed, and often the nonspecific action of the applied drugs like diazoxide is an issue (Hanley et al., 2002, 2005; Hanley and Daut, 2005; Halestrap et al., 2007). It has been proposed that complex II is involved in the composition or regulation of the mitoK_{ATP} channel (Ardehali et al., 2004; Wojtovich and Brookes, 2009). Marban and colleagues isolated a multienzyme complex from rat liver mitochondria that showed pharmacological characteristics of the proposed mitoK_{ATP} channel, contained complex II, and transported potassium when reconstituted into proteoliposomes (Ardehali et al., 2004). Unfortunately, not all proteins of the multienzyme complex were identified, and the identity of the potassium-transporting protein is still obscure. An important study showed that the highly potent and specific complex II inhibitor atpenin A5 had the same cardioprotective effects as ischemic preconditioning, diazoxide, and malonate in cardiomyocytes and in Langendorff-perfused rat hearts (Wojtovich and Brookes, 2009). The authors suggested a functional association of the mitoK_{ATP} channel and complex II, which seems to be supported by the broad pharmacological overlap between the two proteins (Burwell et al., 2009). Although the studies in cardiomyocytes and isolated hearts clearly showing the cardioprotective effect of atpenin A5 represent more physiological conditions than the in vitro study presented in this article, a significant portion of the results in this work were also obtained under in vitro conditions (e.g., an osmotic swelling assay with isolated mitochondria was used to monitor the K_{ATP} channel activity and the inhibition of complex II activity) (Wojtovich and Brookes, 2009). Moreover, complex II activity but not osmotic swelling was assayed in the presence of rather high concentrations of ubiquinone-2, which may have affected the efficiency of a competitive inhibitor like atpenin A5 (Horsefield et al., 2006). Our data suggest an alternative explanation for the cardioprotective effect of the tested complex II inhibitors by direct modulation of complex II activity resulting in ambivalent effects on mitochondrial ROS generation. In line with our proposed model for the production of "signaling ROS" at complex III are in vivo studies with mouse myogenic arteries (Adebiyi et al., 2008); diazoxide induced vasodilation by complex II inhibition and ROS-dependent K_{Ca} and voltage-gated K^+ (K_v) channel activation. Interestingly, atpenin A5 had similar effects. Our model can even explain why an attenuation of the diazoxide effect was observed after application of 1 μM atpenin A5 (Adebiyi et al., 2008): at this high atpenin concentration, complex II is expected to be inhibited completely and the Q-pool will be highly oxidized. As a consequence, little ROS could be produced at the Q_o site of complex III.

Acknowledgments

We thank Ilka Siebels for outstanding technical assistance.

Authorship Contributions

Participated in research design: Dröse and Brandt.
Conducted experiments: Dröse and Bleier.
Performed data analysis: Dröse and Bleier.
Wrote or contributed to the writing of the manuscript: Dröse and Brandt.

References

Adebiyi A, McNally EM, and Jaggar JH (2008) Sulfonylurea receptor-dependent and -independent pathways mediate vasodilation induced by ATP-sensitive K+ channel openers. *Mol Pharmacol* **74:**736–743.
Ardehali H, Chen Z, Ko Y, Mejía-Alvarez R, and Marbán E (2004) Multiprotein complex containing succinate dehydrogenase confers mitochondrial ATP-sensitive K+ channel activity. *Proc Natl Acad Sci USA* **101:**11880–11885.
Borek A, Sarewicz M, and Osyczka A (2008) Movement of the iron-sulfur head domain of cytochrome bc(1) transiently opens the catalytic Q(o) site for reaction with oxygen. *Biochemistry* **47:**12365–12370.
Brand MD (2010) The sites and topology of mitochondrial superoxide production. *Exp Gerontol* **45:**466–472.
Burwell LS, Nadtochiy SM, and Brookes PS (2009) Cardioprotection by metabolic shut-down and gradual wake-up. *J Mol Cell Cardiol* **46:**804–810.
Cape JL, Aidasani D, Kramer DM, and Bowman MK (2009) Substrate redox potential controls superoxide production kinetics in the cytochrome bc complex. *Biochemistry* **48:**10716–10723.
Dröse S and Brandt U (2008) The mechanism of mitochondrial superoxide production by the cytochrome bc1 complex. *J Biol Chem* **283:**21649–21654.
Dröse S, Brandt U, and Hanley PJ (2006) K+-independent actions of diazoxide question the role of inner membrane KATP channels in mitochondrial cytoprotective signaling. *J Biol Chem* **281:**23733–23739.
Dröse S, Hanley PJ, and Brandt U (2009) Ambivalent effects of diazoxide on mitochondrial ROS production at respiratory chain complexes I and III. *Biochim Biophys Acta* **1790:**558–565.
Facundo HT, de Paula JG, and Kowaltowski AJ (2007) Mitochondrial ATP-sensitive K+ channels are redox-sensitive pathways that control reactive oxygen species production. *Free Radic Biol Med* **42:**1039–1048.
Galkin A and Brandt U (2005) Superoxide radical formation by pure complex I (NADH:ubiquinone oxidoreductase) from *Yarrowia lipolytica*. *J Biol Chem* **280:**30129–30135.
Halestrap AP, Clarke SJ, and Khaliulin I (2007) The role of mitochondria in protection of the heart by preconditioning. *Biochim Biophys Acta* **1767:**1007–1031.
Halestrap AP and Pasdois P (2009) The role of the mitochondrial permeability transition pore in heart disease. *Biochim Biophys Acta* **1787:**1402–1415.
Hanley PJ and Daut J (2005) K(ATP) channels and preconditioning: a re-examination of the role of mitochondrial K(ATP) channels and an overview of alternative mechanisms. *J Mol Cell Cardiol* **39:**17–50.
Hanley PJ, Dröse S, Brandt U, Lareau RA, Banerjee AL, Srivastava DK, Banaszak LJ, Barycki JJ, Van Veldhoven PP, and Daut J (2005) 5-Hydroxydecanoate is

metabolised in mitochondria and creates a rate-limiting bottleneck for beta-oxidation of fatty acids. *J Physiol* **562:**307–318.
Hanley PJ, Mickel M, Löffler M, Brandt U, and Daut J (2002) K(ATP) channel-independent targets of diazoxide and 5-hydroxydecanoate in the heart. *J Physiol* **542:**735–741.
Holmuhamedov EL, Jahangir A, Oberlin A, Komarov A, Colombini M, and Terzic A (2004) Potassium channel openers are uncoupling protonophores: implication in cardioprotection. *FEBS Lett* **568:**167–170.
Horsefield R, Yankovskaya V, Sexton G, Whittingham W, Shiomi K, Omura S, Byrne B, Cecchini G, and Iwata S (2006) Structural and computational analysis of the quinone-binding site of complex II (succinate-ubiquinone oxidoreductase): a mechanism of electron transfer and proton conduction during ubiquinone reduction. *J Biol Chem* **281**:7309–7316.
Hunte C, Zickermann V, and Brandt U (2010) Functional modules and structural basis of conformational coupling in mitochondrial complex I. *Science* **329:**448–451.
Kopustinskiene DM, Liobikas J, Skemiene K, Malinauskas F, and Toleikis A (2010) Direct effects of K(ATP) channel openers pinacidil and diazoxide on oxidative phosphorylation of mitochondria in situ. *Cell Physiol Biochem* **25**:181–186.
Kowaltowski AJ, de Souza-Pinto NC, Castilho RF, and Vercesi AE (2009) Mitochondria and reactive oxygen species. *Free Radic Biol Med* **47**:333–343.
Kussmaul L and Hirst J (2006) The mechanism of superoxide production by NADH: ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. *Proc Natl Acad Sci USA* **103**:7607–7612.
Lambert AJ and Brand MD (2004) Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I). *J Biol Chem* **279**:39414–39420.
Liu B, Zhu X, Chen CL, Hu K, Swartz HM, Chen YR, and He G (2010) Opening of the mitoKATP channel and decoupling of mitochondrial complex II and III contribute to the suppression of myocardial reperfusion hyperoxygenation. *Mol Cell Biochem* **337**:25–38.
Miyadera H, Shiomi K, Ui H, Yamaguchi Y, Masuma R, Tomoda H, Miyoshi H, Osanai A, Kita K, and Omura S (2003) Atpenins, potent and specific inhibitors of mitochondrial complex II (succinate-ubiquinone oxidoreductase). *Proc Natl Acad Sci USA* **100**:473–477.
Muller F, Crofts AR, and Kramer DM (2002) Multiple Q-cycle bypass reactions at the Qo site of the cytochrome bc1 complex. *Biochemistry* **41:**7866–7874.
Muller FL, Liu Y, and Van Remmen H (2004) Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J Biol Chem* **279:**49064–49073.
Oldenburg O, Cohen MV, and Downey JM (2003) Mitochondrial K(ATP) channels in preconditioning. *J Mol Cell Cardiol* **35:**569–575.
Ozcan C, Bienengraeber M, Dzeja PP, and Terzic A (2002) Potassium channel openers protect cardiac mitochondria by attenuating oxidant stress at reoxygenation. *Am J Physiol Heart Circ Physiol* **282:**H531–H539.
Pasdois P, Beauvoit B, Tariosse L, Vinassa B, Bonoron-Adèle S, and Dos Santos P (2008) Effect of diazoxide on flavoprotein oxidation and reactive oxygen species generation during ischemia-reperfusion: a study on Langendorff-perfused rat hearts using optic fibers. *Am J Physiol Heart Circ Physiol* **294:**H2088–H2097.
Riess ML, Camara AK, Heinen A, Eells JT, Henry MM, and Stowe DF (2008) KATP channel openers have opposite effects on mitochondrial respiration under different energetic conditions. *J Cardiovasc Pharmacol* **51:**483–491.
Sarewicz M, Borek A, Cieluch E, Swierczek M, and Osyczka A (2010) Discrimination between two possible reaction sequences that create potential risk of generation of deleterious radicals by cytochrome bc1. Implications for the mechanism of superoxide production. *Biochim Biophys Acta* **1797:**1820–1827.
Schäfer G, Portenhauser R, and Trolp R (1971) Inhibition of mitochondrial metabolism by the diabetogenic thiadiazine diazoxide. I. Action on succinate dehydrogenase and TCA-cycle oxidations. *Biochem Pharmacol* **20:**1271–1280.
St-Pierre J, Buckingham JA, Roebuck SJ, and Brand MD (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem* **277:**44784–44790.
Starkov AA (2008) The role of mitochondria in reactive oxygen species metabolism and signaling. *Ann NY Acad Sci* **1147:**37–52.
Sun F, Huo X, Zhai Y, Wang A, Xu J, Su D, Bartlam M, and Rao Z (2005) Crystal structure of mitochondrial respiratory membrane protein complex II. *Cell* **121:** 1043–1057.
Vanden Hoek TL, Becker LB, Shao Z, Li C, and Schumacker PT (1998) Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem* **273:**18092–18098.
Votyakova TV and Reynolds IJ (2001) DeltaPsi(m)-Dependent and -independent production of reactive oxygen species by rat brain mitochondria. *J Neurochem* **79:**266–277.
Wojtovich AP and Brookes PS (2009) The complex II inhibitor atpenin A5 protects against cardiac ischemia-reperfusion injury via activation of mitochondrial KATP channels. *Basic Res Cardiol* **104**:121–129.

Address correspondence to: Dr. Ulrich Brandt, Molecular Bioenergetics Group, Cluster of Excellence Frankfurt "Macromolecular Complexes," Medical School, Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, Haus 26, 60590 Frankfurt am Main, Germany. E-mail: brandt@zbc.kgu.de