

Increased Potassium Conductance of Brain Mitochondria Induces Resistance to Permeability Transition by Enhancing Matrix Volume*

Received for publication, May 7, 2009, and in revised form, October 13, 2009. Published, JBC Papers in Press, October 30, 2009, DOI 10.1074/jbc.M109.017731

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Modulation of K⁺ conductance of the inner mitochondrial membrane has been proposed to mediate preconditioning in ischemia-reperfusion injury. The mechanism is not entirely understood, but it has been linked to a decreased activation of mitochondrial permeability transition (mPT). In the present study K⁺ channel activity was mimicked by picomolar concentrations of valinomycin. Isolated brain mitochondria were exposed to continuous infusions of calcium. Monitoring of extramitochondrial Ca²⁺ and mitochondrial respiration provided a quantitative assay for mPT sensitivity by determining calcium retention capacity (CRC). Valinomycin and cyclophilin D inhibition separately and additively increased CRC. Comparable degrees of respiratory uncoupling induced by increased K⁺ or H⁺ conductance had opposite effects on mPT sensitivity. Protonophores dose-dependently decreased CRC, demonstrating that so-called mild uncoupling was not beneficial *per se*. The putative mitoK_{ATP} channel opener diazoxide did not mimic the effect of valinomycin. An alkaline matrix pH was required for mitochondria to retain calcium, but increased K⁺ conductance did not result in augmented ΔpH. The beneficial effect of valinomycin on CRC was not mediated by H₂O₂-induced protein kinase Cε activation. Rather, increased K⁺ conductance reduced H₂O₂ generation during calcium infusion. Lowering the osmolarity of the buffer induced an increase in mitochondrial volume and improved CRC similar to valinomycin without inducing uncoupling or otherwise affecting respiration. We propose that increased potassium conductance in brain mitochondria may cause a direct physiological effect on matrix volume inducing resistance to pathological calcium challenges.

A short non-injurious ischemic insult can greatly reduce the severity of a subsequent prolonged ischemia, a phenomenon generally referred to as preconditioning or ischemic tolerance (1–3). This adaptive response can be seen as a general biological

phenomenon by which organisms respond with protective mechanisms to potentially recurring challenges (4). A low dose of one type of stressful stimulus can also induce resistance to another, *e.g.* prior transient hyperthermia can protect against subsequent forebrain ischemia (5). Understanding and controlling these seemingly general endogenous survival responses might enable a clinical therapeutic opportunity to reduce tissue damage after cerebral or cardiac ischemia (4, 6, 7). Ischemic preconditioning mediates both a rapid adaptive response coming into effect within minutes to hours as well as an induced tolerance occurring over a longer time frame requiring gene activation and *de novo* protein synthesis. The former is extensively studied in cardiac ischemia and the latter in cerebral ischemia (6, 7). Various physiological and chemical triggers can induce preconditioning, and whereas several mediating pathways have been characterized, the final causal effectors remain more obscure (7).

The inner mitochondrial membrane has been proposed to contain ATP-sensitive potassium channels (mitoK_{ATP}),² which are pharmacologically distinct from plasma membrane K_{ATP} and may mediate as well as be end-effectors of the preconditioning effect. Although there is substantial support for mitoK_{ATP} activation in preconditioning, the evidence comes almost exclusively from studies with pharmacological compounds, mainly diazoxide and 5-hydroxydecanoate (7, 8). Ischemic preconditioning can be closely mimicked by the K_{ATP} opener diazoxide, which shows a concentration-dependent selectivity for mitoK_{ATP} over plasma membrane K_{ATP} (9, 10). Both ischemic and diazoxide-mediated preconditioning can be blocked by 5-hydroxydecanoate, which is believed to inhibit the diazoxide-induced opening of mitoK_{ATP} (11). A role of mitoK_{ATP} is also implicated in different models of cerebral ischemia where pretreatment with diazoxide mediates neuronal protection in a 5-hydroxydecanoate-sensitive manner (12, 13).

Activation of the mitochondrial permeability transition (mPT) phenomenon is considered to participate in cell death pathways associated with various types of pathological stimuli,

* This work was supported by the Swedish Research Council (reference number 2008-2634), by the Japanese Ministry of Health and Labor and Welfare Grant 18591724, by the Swedish Society of Medicine, and by the foundations of Stohne, Segerfalk, and the Royal Physiographic Society in Lund. E. Elmér is co-founder and officer of Maas Biolab, LLC and NeuroVive Pharmaceutical AB, which hold intellectual property rights and develop the use of cyclosporins as cyclophilin D inhibitors for neurological treatment.

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² The abbreviations used are: mitoK_{ATP}, mitochondrial ATP-sensitive potassium channels; mPT, mitochondrial permeability transition; CRC, calcium retention capacity; Debio-025 (UNIL025), D-MeAla³EtVal⁴-cyclosporin; ΔΨ_m, mitochondrial membrane potential; PKCε, protein kinase Cε; ROS, reactive oxygen species; MPG, N-2-mercaptopropionylglycine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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particularly ischemia-reperfusion injury (14–16). The mPT is also implicated in the pathogenesis of several acute or chronic neurodegenerative diseases, substantiated by the neuroprotective effects of cyclosporin A, its non-immunosuppressive analogs, or genetic deletion of the mPT component and cyclosporin A target cyclophilin D in animal models (17–19).

Ischemic preconditioning and mito K_{ATP} channel activation have been proposed to afford tissue protection by inhibiting mPT activation during reperfusion (8, 10, 20, 21). A signaling pathway linking mito K_{ATP} and mPT has been proposed where the increased K^+ conductance after mito K_{ATP} opening alkalinizes the mitochondrial matrix and increases generation of H_2O_2 , which in turn activates an mPT-associated PKC ϵ (22, 23). An alkaline matrix pH has also been put forward as the basis for efficient mitochondrial calcium buffering (24). Furthermore, prevention of matrix contraction by K^+ -mediated volume effects may preserve mitochondrial respiratory capacity and energy transfer during ischemia-reperfusion (25–27).

The aims of the present study were to (i) investigate if increased K^+ conductance modulates calcium retention capacity (CRC) and activation of mPT in brain mitochondria and (ii) to explore the physiological effects of increased K^+ conductance and determine the mechanism by which activation of mitochondrial K^+ channels improves mitochondrial resistance to calcium challenges in neuronal preconditioning.

EXPERIMENTAL PROCEDURES

Isolation of Brain Mitochondria—Adult male Wistar rats (Harlan Scandinavia ApS, Allerød, Denmark) of 325–400 g were allowed *ad libitum* access to water and food before use. Animals were normally decapitated after a brief exposure to halothane to minimize stress, but one animal for each experimental setting was decapitated without gaseous anesthesia to ensure that the process did not influence the results. All animal procedures were approved by the Malmö/Lund Ethical Committee for Animal Research (M221-03, M44-07). Isolation of cortical non-synaptosomal brain mitochondria was achieved by using a discontinuous Percoll gradient as described previously (28, 29). All steps were carried out under ice-cold conditions. Protein quantification of mitochondrial suspensions was performed by the Bradford method using bovine serum albumin as standard.

Mitochondrial CRC, NAD(P)H Fluorescence, Membrane Potential ($\Delta\Psi_m$), Light Scattering, and H_2O_2 Generation—A luminescence spectrometer LS-50B (PerkinElmer Life Sciences) with a temperature controlled cuvette holder (37 °C) was used for all fluorescence and light-scattering experiments. Mitochondria (100 μ g/2 ml) were suspended in 125 mM KCl, 20 mM Tris buffer, pH 7.1, containing 2 mM P_i (K^+), 1 mM $MgCl_2$, 1 μ M EGTA, 200 μ M ATP, and 5 mM NADH-linked respiratory substrates malate and glutamate. After the addition of mitochondria, 1 μ g/ml oligomycin, 50 μ M ADP, and experimental compounds were added. In experiments evaluating matrix volume effects on CRC, the osmolarity of the buffer was decreased by lowering the KCl concentration from 125 to 75 mM. Mitochondrial calcium uptake and release were monitored by the excitation ratio (excitation 340/380 nm, emission 509 nm) of the extramitochondrial calcium-sensitive fluorescent probe

Fura 6F (250 nm). The mitochondrial suspensions were infused with 200 nmol of $CaCl_2/(mg \times min)$ (10 μ M/min). The start of calcium uptake was defined as the point where the experimental curve deviated from a control curve with 1 μ M ruthenium red, blocking mitochondrial calcium uptake through the uniporter. CRC was calculated as the amount of infused calcium from the start of mitochondrial calcium uptake until the start of maximal calcium release. The redox status of NAD(P)H was determined qualitatively by following autofluorescence of NAD(P)H with excitation at 340 nm and emission at 460 nm during calcium infusions. $\Delta\Psi_m$ was assessed qualitatively by following fluorescence quenching of rhodamine 123 (100 nm), excitation and emission at 490 and 528 nm. Light scattering of mitochondria was detected at 90° and measured at 520 nm. Mitochondrial H_2O_2 generation was detected by following the oxidation of 1 μ M Amplex Red to the fluorescent product resorufin in the presence of horseradish peroxidase and superoxide dismutase (0.5 and 20 units/ml, respectively). Excitation was set to 560 nm and emission to 590 nm. Known amounts of H_2O_2 were added to establish a calibration curve.

High Resolution Respirometry—Oxygen consumption of mitochondria during calcium infusion was measured under similar conditions as Fura 6F experiments described above except for the continuous decline in oxygen concentration in the sealed chamber. Also, 10 μ M bovine serum albumin was present in the buffers of the experiments comparing respiration and CRC in 75 and 125 mM KCl. Studies were performed in an Oroboros Oxygraph-2k with a Titration-Injection microPump TIP-2k using DatLab 4 software allowing online respiration rate output with high sensitivity, low noise, and concentration-dependent background correction (Oroboros Instruments, Innsbruck, Austria) (30). CRC in the respiration experiments was calculated as the amount of infused calcium from the start of elevated respiration rate until the start of rapid respiration decrease.

Matrix pH Measurements—Changes in intramitochondrial pH were measured by determining the equilibrium distribution of the weak acid acetate (31). The uncharged protonated species (HAc) equilibrates over the mitochondrial membranes. The anion (Ac^-) is impermeant and, therefore, accumulates in an alkaline compartment, such as the mitochondrial matrix, due to continued dissociation and uptake of HAc. The pH difference can be determined by the relationship $\Delta pH = \log_{10} Ac_{ext}^-/Ac_m^-$, where ext and m refers to extra-matrix and matrix compartments, respectively. A whole preparation of brain mitochondria (~700 μ g) was divided in half and incubated in the ordinary KCl media supplemented with 3H -labeled acetate (0.5 μ Ci or 18.5 kBq/ml) and ^{14}C -labeled sucrose (0.05 μ Ci or 1.85 kBq/ml) and 1 mM “cold” acetate and sucrose. [^{14}C]Sucrose does not permeate the inner mitochondrial membrane and was used to correct for non-matrix 3H activity. After incubation with either 3 pmol/mg valinomycin or vehicle (ethanol) in the presence of adenonucleotides and respiratory substrates (see above), the suspension was carefully layered on top of 900 μ l of silicone oil AR 110 and centrifuged at 20,800 $\times g$ for 2 min, leaving the mitochondria in a pellet and the aqueous phase above the silicone oil. Half of the supernatant (500 μ l) was transferred to new vials. The remaining supernatant and sili-

cone oil was carefully but rapidly removed, and the mitochondrial pellet was lysed in 500 μ l of H_2O . One series of experiments was also performed without filtering through silicone oil. Both pellet and supernatant samples were deproteinized with perchloric acid (2% w/v final) (32), and the precipitate was spun down. Two samples (2 μ l) of supernatant and 500 μ l of the clear pellet solution were transferred to scintillation vials (HDPE 24 ml, VWR) with 5 ml of Ready Safe scintillation fluid (Beckman Coulter, Fullerton, CA) and H_2O (10:1 final). Samples were measured in a LS 6500 Scintillation counter (Beckman Coulter). Similar experiments were performed with [3H] H_2O for matrix volume estimates but did not yield reliable values as the signal to noise ratio was far inferior to that of tritiated acetate. For Δ pH calculations, a matrix volume of 1 μ l/mg was assumed (33).

Statistical Analyses—All experiments were replicated in at least four separate mitochondrial preparations. Average results are presented as the mean \pm S.D. and were evaluated with one-way ANOVA followed by the Bonferroni post hoc test. The level of statistical significance was set to 5%.

RESULTS

Increased K^+ Conductance Induced by Low Concentrations of Valinomycin Increases the CRC of Brain Mitochondria—The lipophilic K^+ carrier valinomycin induces an increased K^+ conductance of the inner mitochondrial membrane and translocates K^+ from the intermembrane side to the matrix side electrophoretically as a result of the mitochondrial membrane potential ($\Delta\Psi_m$, inside negative) (34). In the present study, valinomycin was administered at low concentrations to mimic K^+ channel activity, and the effect on physiological parameters of brain mitochondria such as oxygen consumption, $\Delta\Psi_m$, light scattering, NAD(P)H fluorescence, and the ability to sequester calcium was assayed. Valinomycin at 150 μ M (3 pmol/mg of mitochondria) significantly increased the CRC of brain mitochondria exposed to a continuous infusion of calcium (Figs. 1 and 3). Parallel to the CRC assay, during calcium infusion, valinomycin prolonged the delay before a rapid decrease of NAD(P)H fluorescence and oxygen consumption (data not shown and Fig. 2, respectively). The dose-response relationship demonstrated a significant increase of CRC at 150–300 μ M, whereas higher concentrations gradually attenuated the positive effect, and 2 nM valinomycin decreased CRC (Fig. 3).

Increased H^+ Conductance by the Protonophore CCCP or Electroneutral K^+/H^+ Exchange by Nigericin Reduces the Calcium Retention Capacity of Brain Mitochondria—In contrast to the effect of valinomycin, increased H^+ conductance of the inner mitochondrial membrane by the protonophore CCCP dose-dependently decreased CRC over all tested concentrations and completely abolished CRC at 200 nM (Fig. 3). The lowest tested concentrations of CCCP (25 nM) and valinomycin (150 μ M) both significantly increased basal respiration rate from control (1454.1 ± 202 , 1449.5 ± 257 , and 647.6 ± 150 pmol $O_2/(s \times mg)$, respectively), and there was no significant difference between the compounds (Fig. 2). Thus, even though the compounds displayed similar degrees of uncoupling, they exhibited opposite effects on CRC. The electroneutral K^+/H^+

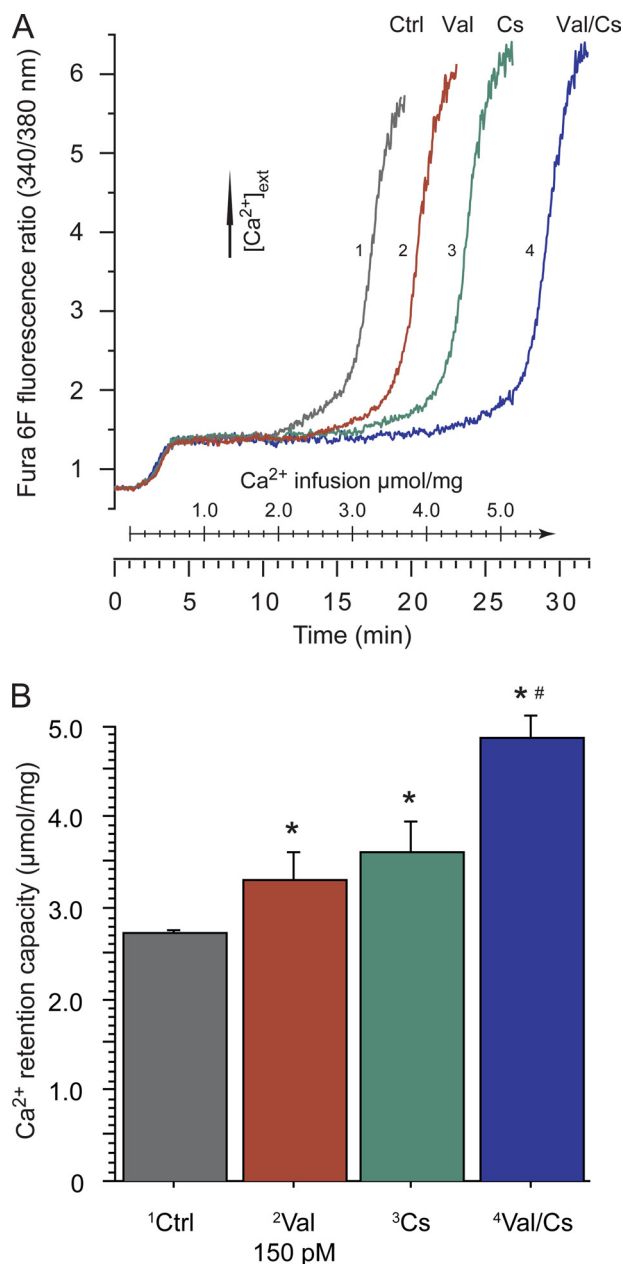


FIGURE 1. Increased K^+ conductance by valinomycin and inhibition of cyclophilin D separately and additively increase brain mitochondrial calcium retention capacity. Suspensions of isolated brain mitochondria in KCl-based buffer (100 μ g/2 ml) were infused with 200 nmol of $Ca^{2+}/(mg \times min)$ (10 μ M Ca^{2+}/min). Experiments were run in the presence of 200 μ M ATP, 1 μ g/ml oligomycin, 50 μ M ADP, and 5 mM malate and glutamate. Additions were made with 150 μ M K^+ carrier valinomycin (Val) and/or 1 μ M non-immunosuppressive cyclosporin analog D-MeAla³EtVal⁴-cyclosporin (Cs, also called Debio-025 or UNIL025). Vehicle (0.2% ethanol) was present in control runs (Ctrl). A, CRC of brain mitochondria was determined by following the fluorescence ratio of Fura 6F (250 nm) reflecting extramitochondrial Ca^{2+} concentration. A plateau level is reached when mitochondrial calcium uptake equals the rate of infused calcium, and rapid calcium release occurs at a certain threshold considered to reflect activation of mPT. B, CRC was calculated as the amount of infused calcium from the start of calcium uptake (the time point where the experimental curve deviated from a control curve with inhibited calcium uptake) to the start of maximal calcium release. Values are the means \pm S.D. (μ mol Ca^{2+}/mg mitochondria) of four separate mitochondrial preparations. The asterisk (*) indicates significant difference compared with control (vehicle run), and the number symbol (#) indicates significant combined effect of the valinomycin and D-MeAla³EtVal⁴-cyclosporin compared with each of the drugs separately.

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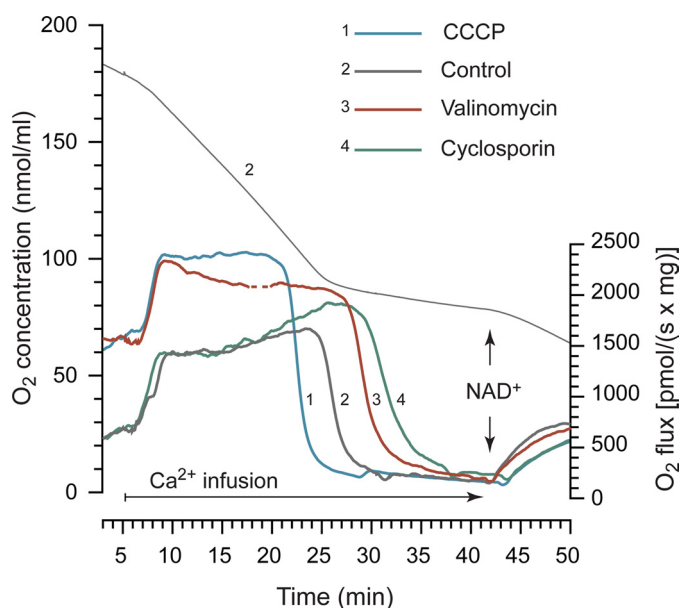


FIGURE 2. High resolution respirometry of brain mitochondria during calcium uptake and release. Experiments were performed as described in Fig. 1, except mitochondrial suspensions were analyzed in air-tight chambers. Oxygen consumption of mitochondria was determined during calcium infusion, and the *upper trace* displays the O_2 concentration (nmol/ml) in the chamber for a representative control experiment. The *lower traces* show the real-time O_2 consumption rate ($\text{pmol } O_2/\text{s} \times \text{mg mitochondria}$) of vehicle (Control), 150 μM valinomycin, 1 μM D-MeAla³EtVal⁴-cyclosporin, or 25 nM protonophore CCCP. Basal respiration rates were significantly higher in the presence of valinomycin or CCCP. In all runs calcium infusion induced an increased rate of respiration from basal levels followed by a rapid decrease in respiration, attributed to permeability transition. To confirm altered inner membrane permeability, 150 μM NAD^+ was administered, which increased respiration in all groups after calcium infusion. Traces are representative examples of four separate experiments. Disturbances because of air inlet in the chamber have been deleted in trace 3 (*dashed segment*). Quantification of CRC is provided in Figs. 1 and 3.

exchanger nigericin (5 nM) hyperpolarized the mitochondria (data not shown) and significantly decreased the CRC of brain mitochondria suspended in a buffer with physiological pH (Fig. 5A). An increase in $\Delta\Psi_m$ was, therefore, not sufficient to increase CRC *per se*.

Permeability Transition Limits the Extent of Mitochondrial Calcium Retention—mPT is classically assayed in light-scattering assays measuring matrix volume or configuration changes. Activation of mPT is usually detected after large bolus additions of calcium or by calcium loading followed by a second inducing agent such as an oxidant or protonophore. Although the mitochondria behave as though they have certain individual probabilities to undergo mPT, after a large bolus of calcium the mPT becomes more of an all-or-nothing phenomenon for the whole mitochondrial population. A compound that slightly influences the threshold for mPT activation or interferes with the calcium loading can, therefore, display a dramatic effect. Recently, more quantitative assays have been introduced where either small repetitive calcium doses or, as in the present study, a continuous infusion of calcium is administered. The strengths of these latter assays are a lesser degree of sudden bioenergetic demand for the mitochondria (24) and a quantitative evaluation of both positive and negative effects of a tested treatment. The measurement of extramitochondrial calcium provides a functional assay for mitochondrial calcium uptake (Fig. 1A). The

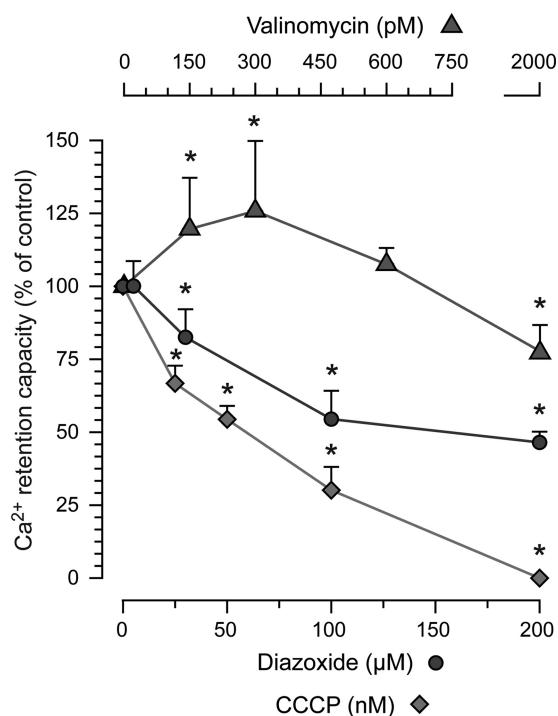


FIGURE 3. Dose-related effects of increased K^+ conductance and H^+ conductance or diazoxide on brain mitochondrial calcium retention. Experiments were run as described in Fig. 1, and CRC is expressed as % of control (vehicle, 0.2% ethanol or DMSO). The K^+ carrier valinomycin increased CRC at low concentrations, but a decrease was seen at the highest tested concentration. In contrast, all tested concentrations of the protonophore CCCP dose-dependently decreased CRC. The K^+ -ATP channel opener diazoxide was without effect at 5 μM , but higher concentrations decreased CRC. The asterisk indicates significant difference compared with control.

rapid calcium release after a prolonged period of calcium uptake occurs concomitantly with a decrease in light-scattering and $NAD(P)H$ fluorescence in brain mitochondria and has, therefore, been attributed to activation of mPT (24). However, the calcium-phosphate complexes formed within the matrix during calcium loading interfere with light-scattering and $NAD(P)H$ fluorescence, and changes in these parameters are not necessarily associated with mPT (35). To obtain a more specific correlation between CRC and mPT, the calcium infusion experiments were repeated in airtight chambers during measurements of oxygen consumption (Fig. 2). Mitochondrial respiration increased during active calcium uptake and retention, but at the time, corresponding to rapid calcium release, respiration was severely decreased. The respiratory inhibition was attributed to loss of $NAD(H)$ from the matrix after mPT. The addition of exogenous NAD^+ , which is impermeant to the inner mitochondrial membrane in intact mitochondria, increased respiration after calcium infusion, indicating that the mitochondria had undergone mPT (Fig. 2). It was not necessary to supplement cytochrome *c* in these experiments to reveal the stimulation of respiration by NAD^+ after mPT, but it may be necessary in mitochondria isolated from other organs (36). Further identifying the mPT as the limiting factor for mitochondrial CRC is the finding that the cyclophilin D inhibitor D-MeAla³EtVal⁴-cyclosporin (Debio-025) increased CRC independently and additively to valinomycin (Figs. 1 and 2).

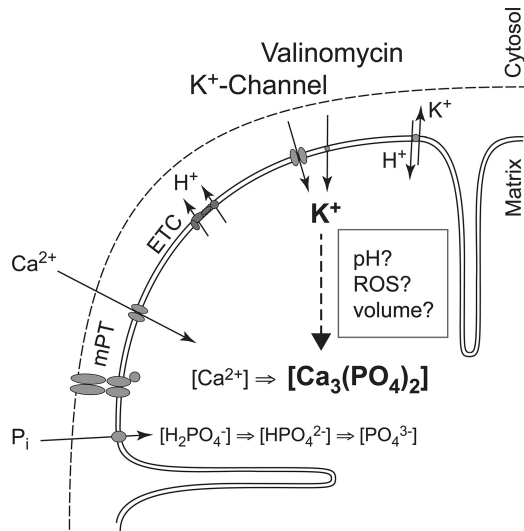


FIGURE 4. How increased K^+ conductance may improve mitochondrial calcium retention and sensitivity to permeability transition. Schematic illustrations are shown of some proposed physiological effects caused by increased K^+ conductance under conditions where mitochondria buffer an increased cytoplasmic calcium load. Increased K^+ conductance by K^+ channel activation or by the K^+ -carrier valinomycin may enhance matrix alkalization via increased H^+ extrusion by the electron transport chain (ETC). The uptake of phosphate (P_i) and the equilibrium between differently protonated phosphate groups each depends on pH. An alkaline pH enhances the availability of PO_4^{3-} , enabling formation of calcium phosphate complexes (e.g. $Ca_3(PO_4)_2$) from free Ca^{2+} . Enhanced matrix volume due to the osmotic effect of K^+ may also lead to increased availability of P_i and enhanced calcium complex formation. The maintenance of low $[Ca^{2+}]$ in the mitochondrial matrix reduces the sensitivity of mPT and may, therefore, increase CRC. Increased K^+ conductance has further been suggested to elevate mitochondrial ROS production, which activates PKC ϵ and, through its claimed association with the mPT pore complex, induces resistance to mPT (22, 44, 53).

Calcium Retention Capacity of Brain Mitochondria Is Dependent on an Alkaline Matrix pH—A low pH is considered to stabilize the mPT pore complex in a closed configuration. This is apparent in mPT assays run under de-energized conditions (37, 38), *i.e.* under conditions where active calcium uptake and retention is inhibited. The beneficial effect of acidic pH in inducing resistance toward mPT was confirmed for de-energized brain mitochondria in the present study (data not shown) and has also been demonstrated by others (39). In contrast, under physiological conditions, an alkaline mitochondrial matrix is considered to be the basis for efficient calcium complexation with phosphate forming inactive calcium phosphates and, thus, keeping free $[Ca^{2+}]$ at a regulated low level (40) (Fig. 4). To further study the role of pH on mitochondrial calcium accumulation and resistance to mPT, calcium infusion experiments were run over a wide pH range (Fig. 5A). An acidic pH (pH 6.6) was detrimental for CRC, as were extreme alkaline conditions (pH 8.6). Neutral or slightly alkaline conditions were most favorable for the retention of calcium in brain mitochondria (pH 7.1–7.6). In contrast, mitochondria with a reduced ΔpH (treated with the electroneutral K^+/H^+ exchanger nigericin) displayed the largest CRC at pH 7.6–8.1. Thus, with a nigericin-induced reduction in ΔpH , a more alkaline pH is required for efficient calcium retention.

Increased K^+ Conductance Induced by Valinomycin Does Not Cause Increased Alkalization or Reduced Free $[Ca^{2+}]$ in the Mitochondrial Matrix—One possible mechanism whereby increased K^+ conductance can enhance CRC is elevation of

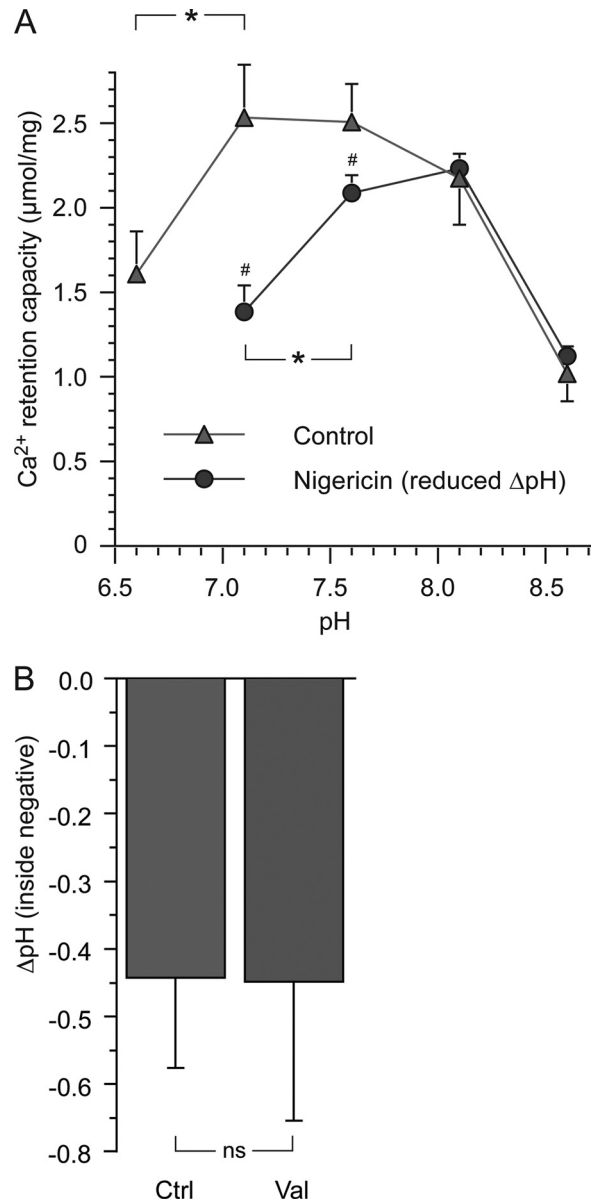


FIGURE 5. The influence of pH on mitochondrial calcium retention and measures of ΔpH . A, experiments were performed as in Fig. 1. The pH of the buffer was modified between 6.6 to 8.6, and for indicated groups, the pH gradient over the inner mitochondrial membrane (ΔpH , normally matrix alkaline) was reduced by the electroneutral K^+/H^+ exchanger nigericin (5 nM). A neutral or slightly alkaline pH was optimal for CRC in control mitochondria, whereas pH 7.6–8.1 was most beneficial for mitochondria with reduced ΔpH . The asterisk (*) indicates significant difference between groups, and the number symbol (#) indicates significant difference of nigericin compared with control. B, shown are calculations of ΔpH using equilibrium distribution of tritiated acetate. Calculations were based on quench-corrected and ^{14}C -subtracted 3H dpm in mitochondrial supernatants or pellets from mitochondria incubated with $[^3H]$ acetate and $[^{14}C]$ sucrose with or without 3 pmol/mg valinomycin (Val). ns indicates no significant difference in ΔpH between groups ($n = 12$).

matrix pH (Fig. 4). A more alkaline matrix lowers free $[Ca^{2+}]$ through increased uptake and deprotonation of phosphate with a resulting increase in calcium-phosphate complexation. To test this hypothesis, intramitochondrial pH was measured in a repeated series of experiments using equilibrium distribution of tritiated acetate. The radioactivity from tritiated acetate used to calculate ΔpH was not significantly different in control and

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valinomycin-treated mitochondria. Experiments where mitochondria were filtered through silicone oil yielded somewhat higher values of ΔpH than experiments without filtration (-0.54 ± 0.12 and -0.39 ± 0.11 for the respective control runs). A summary of the results with all experiments included is presented in Fig. 5B.

Furthermore, there was no indication that increased K^+ conductance caused reduced matrix $[Ca^{2+}]$. Extramitochondrial $[Ca^{2+}]$ was not affected during calcium loading (Fig. 1). In calcium set-point experiments (40), the calcium infusion was halted, and extramitochondrial $[Ca^{2+}]$ reached a new somewhat lower equilibrium level (data not shown). After the addition of small amounts of the calcium chelator EGTA in the presence of 18 mM NaCl, mitochondria gradually released Ca^{2+} and regained a similar equilibrium level of extramitochondrial $[Ca^{2+}]$ as before calcium chelation, *i.e.* the mitochondrial calcium set-point concentration (data not shown). As the activity of mitochondrial Na^+/Ca^{2+} exchanger is dependent on ΔpH and matrix $[Ca^{2+}]$ (41, 42), a difference in these parameters between control and valinomycin-treated mitochondria would expectedly influence the calcium set-point concentration as well as the rate of efflux, but no such differences were observed.

Increased Calcium Retention by Valinomycin Is Not Dependent on Increased ROS and the Associated Activation of PKC ϵ —Garlid and co-workers (23) have described a pathway in isolated heart mitochondria by which increased K^+ conductance through the opening of $mitoK_{ATP}$ or by valinomycin triggers an increase in H_2O_2 . Hydrogen peroxide in turn activates PKC ϵ , which associates with and inhibits mPT. They showed that openers of $mitoK_{ATP}$ or valinomycin inhibited calcium-induced mPT, but the protection could be blocked by the PKC inhibitor Ro318220 and the ROS scavenger *N*-2-mercapto-propionylglycine (MPG) (23). In the present study, Ro318220 (0.1 μM) did not influence the valinomycin-mediated increase of brain mitochondrial CRC (Fig. 6A). MPG (300 μM) decreased CRC both in control and valinomycin-treated mitochondria, whereas the antioxidant catalase (2000 units/ml) had no effect, indicating an unspecific disadvantageous effect of MPG unrelated to ROS scavenging. To further study a possible contribution of ROS generation to the beneficial effect of valinomycin on CRC, H_2O_2 generation was monitored during the calcium infusion experiments (Fig. 6B). The oxidation rate of Amplex Red to the fluorescent product resorufin was significantly decreased in the presence of 150 μM valinomycin. Furthermore, successive additions of 50 μM (1 pmol/mg of mitochondria) valinomycin to brain mitochondria without infused calcium lowered basal H_2O_2 generation in a dose-dependent manner (data not shown). Hence, increased ROS production and activation of PKC ϵ did not mediate the increased capacity of brain mitochondria to accumulate calcium in the present study.

Increasing Matrix Volume Enhances Calcium Retention Capacity without Affecting Respiratory Activity—To investigate effects on matrix volume by increased K^+ conductance and to separate the volume effects from depolarization and uncoupling in relation to CRC, the osmolarity of the suspending buffer was reduced (by reducing the KCl concentration from 125 to 75 mM). Volume effects were estimated by assaying 90° light scattering, and the nonspecific ionophore alamethicin was

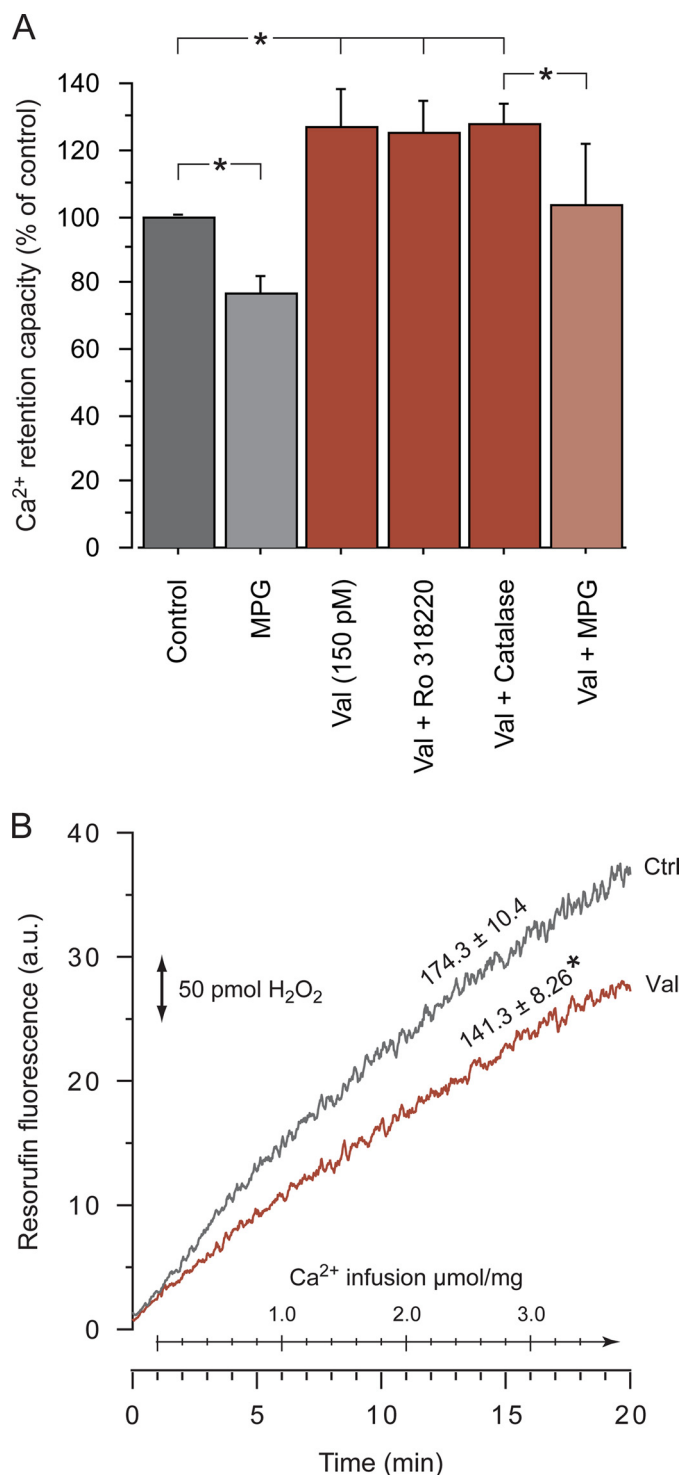


FIGURE 6. Increased calcium retention by valinomycin is not blocked by PKC inhibition or associated with increased H_2O_2 generation. A, shown is CRC of brain mitochondria during a continuous calcium infusion as described in Fig. 1. Mitochondria with 150 μM valinomycin were pretreated with the PKC inhibitor Ro318220 (100 nM), the free radical scavenger MPG (300 μM), or 2000 units/ml of the antioxidant catalase. MPG reduced CRC with and without valinomycin, indicating a nonspecific negative effect. The asterisks indicate significant difference between groups. B, H_2O_2 -dependent oxidation of Amplex Red to the fluorescent product resorufin was followed during calcium infusion for control (Ctrl) and valinomycin-treated (Val, 150 μM) brain mitochondria. Inserted numbers display mean H_2O_2 generation rate in pmol/min/mg. The asterisk indicates significant difference compared with control. a.u., arbitrary units.

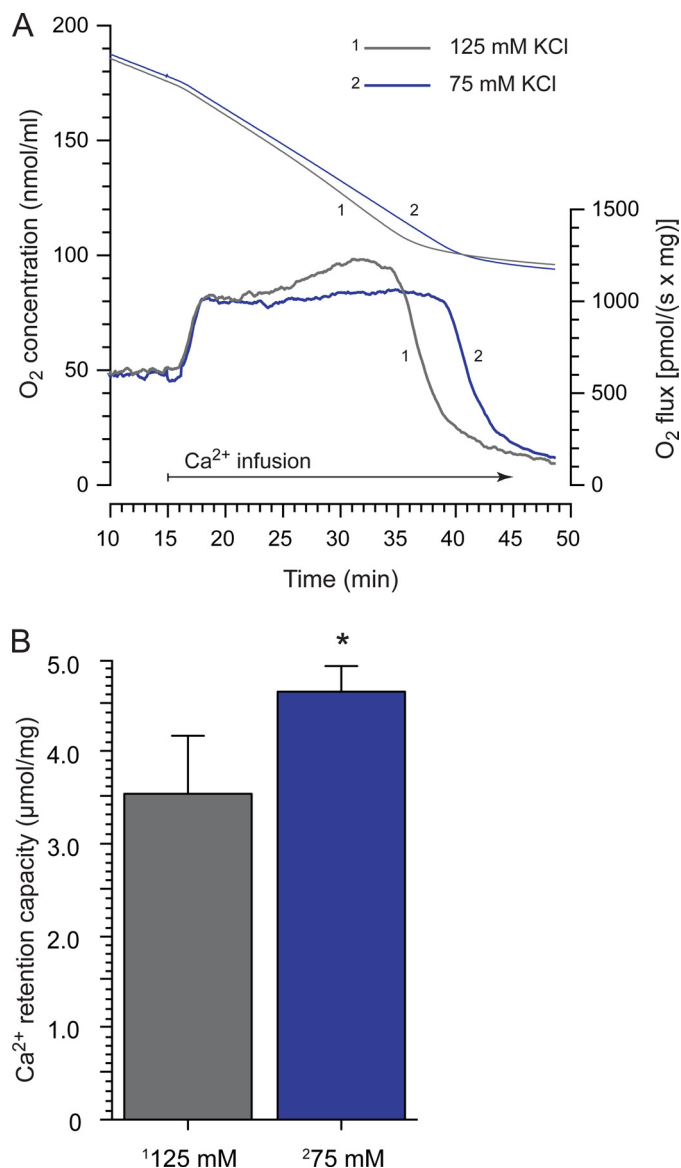


FIGURE 7. The influence of matrix volume on brain mitochondrial calcium retention. Conditions were as described in Figs. 1 and 2 except that $10 \mu\text{M}$ bovine serum albumin was present. *A*, respirometry of mitochondria suspended in standard (125 mM, trace 1) or reduced (75 mM, trace 2) concentrations of KCl during a continuous calcium infusion are shown. The KCl concentration was reduced to lower the medium osmolarity and thereby increase matrix volume. *Upper traces* are representative examples of changes in O_2 concentration, and *lower traces* depict the corresponding respiration rates ($n = 5$). The rapid decrease in respiration rate indicates activation of permeability transition. There were no significant differences in basal or calcium-stimulated respiration rates, but there was significant difference in time to respiratory inhibition. *B*, quantification of CRC of brain mitochondria suspended in 125 or 75 mM KCl media. CRC was calculated as the amount of infused calcium from the start of the elevated respiration rate until start of rapid respiration decrease. The asterisk indicates significant difference between groups.

used to obtain a standardized maximal swelling response (data not shown). The reduced KCl concentration and 150 pM valinomycin, both, reduced light scattering significantly (12.76 ± 2.2 and $17.1 \pm 7.1\%$, respectively, $n = 5-8$) with no significant difference between the treatments. Calculations of CRC demonstrated a significant increase in CRC in mitochondria suspended in 75 mM compared with 125 mM KCl (Fig. 7). The reduced KCl concentration did not, however, significantly

affect respiratory rates before or during calcium uptake ($n = 5$, representative traces demonstrated in Fig. 7A).

The increase in respiration induced by valinomycin was attributed to K^+ cycling and not to volume differences (neither nigericin nor the changes in osmolarity above influenced respiration). The augmented oxygen consumption during mitochondrial calcium accumulation was largest at the start of infusion with a gradual decline corresponding to a K^+ flux of 900 to $350 \text{ nmol}/(\text{min} \times \text{mg})$. Similar flux rates were obtained in the absence of active calcium uptake.

The MitoK_{ATP} Channel Opener Diazoxide Does Not Mimic the Effect of Valinomycin and Reduces Calcium Retention—The putative mitoK_{ATP} channel opener diazoxide was tested at $5-200 \mu\text{M}$ for effects on CRC. Care was taken to follow previously reported conditions for increasing probability of mitoK_{ATP} detection, *i.e.* ATP was present in solution before mitochondria and diazoxide dissolved in DMSO was added immediately after mitochondria to ensure even distribution (22). However, one discrepancy was the time frame of the present experiments compared with typical assays detecting mitoK_{ATP}, which usually monitor the first few minutes after the addition of mitochondria from isolation medium to experimental buffer (22). Diazoxide did not induce any light-scattering changes, in contrast to valinomycin, which induced readily detectable decreases already at 50 pM (data not shown). Whereas $5 \mu\text{M}$ diazoxide had no effect on mitochondrial calcium handling, $30-200 \mu\text{M}$ dose-dependently decreased CRC (Fig. 3). The negative effects of diazoxide were probably related to unspecific effects on mitochondria such as uncoupling of mitochondria at higher doses (43).

Heart Mitochondria—To evaluate the generability of the findings in brain mitochondria, key experiments were performed in isolated heart mitochondria. A similar correlation between matrix volume and CRC was found where an increased matrix volume increased CRC. Aggravated respiratory uncoupling dose-dependently reduced CRC, but the relative importance of uncoupling *versus* volume changes on CRC differed between brain and heart mitochondria (data to be published elsewhere).

DISCUSSION

The main finding of the present study was that increased K^+ conductance, modeled by low concentrations of valinomycin, increases mitochondrial CRC and resistance to mPT in isolated brain mitochondria. Furthermore, CRC was improved by reducing the osmolarity of the surrounding buffer, indicating that the beneficial effect of increased K^+ conductance could be attributed to a direct physiological effect of an enhanced mitochondrial matrix volume.

How Increased K⁺ Conductance May Improve Mitochondrial Calcium Retention and Sensitivity to Permeability Transition—Increasing mitochondrial K^+ conductance by valinomycin or by activation of a K^+ channel (such as the putative mitoK_{ATP}) will cause K^+ to enter mitochondria along its electrochemical gradient. The drop in membrane potential will accelerate NADH oxidation at complex I to rebuild the proton motive force. Increased activity of the K^+/H^+ exchanger will partially counteract the influx of K^+ , and depending on the relative

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increase of K^+ conductance *versus* K^+ extrusion, a new equilibrium will be reached. The previously proposed physiological effects of this process, besides a reduction in $\Delta\Psi_m$ and increased respiration, are (i) matrix alkalization due to the K^+/H^+ exchange, (ii) increased mitochondrial ROS production, which in turn may activate endogenous protective pathways, and (iii) matrix swelling caused by the osmotic effect of K^+ (22, 44) (Fig. 4).

Matrix pH and Calcium Retention—Mitochondria take up calcium electrophoretically through the uniporter above a certain external $[Ca^{2+}]$, the so-called calcium set-point (40). The calcium uptake capacity of mitochondria is very large in the presence of physiological levels of ADP and P_i , but the intramitochondrial free $[Ca^{2+}]$ is kept low because of formation of inactive calcium phosphates, e.g. $Ca_3(PO_4)_2$. The complexation of calcium requires PO_4^{3-} , and both the uptake of $H_2PO_4^-$ and the successive deprotonations to PO_4^{3-} vary with matrix pH. The free $[Ca^{2+}]$ in equilibrium with $Ca_3(PO_4)_2$ would, thus, vary significantly in relation to matrix pH due to P_i availability. A theoretic example for $[Ca^{2+}]$ in equilibrium with $Ca_3(PO_4)_2$ in the presence of 2 mM P_i has been presented by D. G. Nicholls (40), where $[Ca^{2+}]$ would increase from 2 to 100 μM if the matrix pH was reduced from 7.8 to 7.0. Thus, a low matrix pH and P_i content will have a dramatic effect on calcium-phosphate formation, and free matrix $[Ca^{2+}]$ may be elevated to an extent where mPT activation will occur. Our results demonstrate that an expected reduced ΔpH , *i.e.* decreased matrix pH, resulted in a reduced CRC regardless if it occurred concomitantly to an increased $\Delta\Psi_m$ (as with nigericin) or a decreased $\Delta\Psi_m$ (as with CCCP). Furthermore, when external pH was manipulated in mitochondria with or without nigericin treatment, an alkaline matrix pH (up to a certain extent) was coupled to a high CRC, whereas an acidic to neutral matrix pH resulted in low CRC. In de-energized mitochondria, where respiration and consequently $\Delta\Psi_m$ -dependent calcium transport is inhibited and/or calcium transport is facilitated by a calcium-ionophore, low pH is a potent inhibitor, and P_i is a main inducer of mPT (37, 38, 45), and this is the general view of their role in mPT regulation (46, 47). We demonstrate using high resolution respirometry during calcium infusion that mPT is the end point of mitochondrial calcium retention. The determination of CRC and the simultaneous monitoring of O_2 flux offer reliable and quantitative assays for mPT in a physiological context where both negative and positive effects of a given treatment are taken into account. The strong pH dependence of brain mitochondria to buffer external calcium loads demonstrate that the effects on free matrix $[Ca^{2+}]$ by pH and P_i during active mitochondrial calcium loading are more important than their effect on the mPT pore components *per se*.

Several previous studies using isolated mitochondria have demonstrated that valinomycin can increase matrix pH (22, 44, 48, 49). On the other hand, in a physiological medium, permeant anions such as P_i , which are translocated into mitochondria depending on ΔpH , may buffer a matrix pH elevation induced by K^+ flux (48). P_i may also be the main charge-compensating anion accompanying mitochondrial K^+ uptake as the mitochondrial matrix volume increases. Indeed, we demonstrate here in equilibrium distribution measurements of triti-

ated acetate using a P_i substrate, and adenine nucleotide-containing buffer, that no change in ΔpH or calcium set-point occurs after valinomycin treatment. Thus, increased K^+ conductance did not mediate its beneficial effect on CRC through an augmented ΔpH .

Mitochondrial ROS Generation and PKC ϵ Activation—Activation of PKC ϵ is considered to be a key mediator of ischemic preconditioning in the heart (50, 51). Besides a role in activating mitoK_{ATP} (52), PKC ϵ has been shown to associate with and inhibit activation of the mPT pore complex (53). Increased mitochondrial K^+ conductance has been suggested to increase mitochondrial ROS production both *in vitro* and *in vivo* (44, 54). PKC ϵ is believed to be activated by the elevated ROS, desensitizing mPT to activation by calcium (23). The increased ROS has been proposed to be a direct effect of mitoK_{ATP}- or valinomycin-induced alkalization of matrix pH (44). However, in our hands valinomycin consistently decreased detected H_2O_2 in a dose-dependent fashion, and the K^+ carrier significantly lowered the generation of H_2O_2 during calcium infusion, likely related to the depolarization induced by the compound. Furthermore, inhibition of PKC ϵ and H_2O_2 scavenging did not inhibit the beneficial effect of valinomycin. Thus, we conclude that this pathway does not seem to be active in our mitochondrial preparations and does not underlie the beneficial effect of increased K^+ conductance.

Matrix Volume and Calcium Retention—In contrast, increasing matrix volume by lowering the KCl concentration of the surrounding medium resulted in an enhanced CRC similar to that induced by valinomycin, demonstrating that the beneficial effect of increased K^+ conductance in brain mitochondria is probably mediated by an enhanced matrix volume. Respiration and energy transfer can be modulated by matrix volume (25, 26), but no difference in respiration was observed in mitochondria suspended in 75 or 125 mM KCl. Extramitochondrial osmolarity has been shown to influence the sensitivity of liver mitochondria to mPT, which was attributed to volume-dependent concentrations of endogenous mPT modulators (55). Changes in matrix volume have also been demonstrated to alter the mitochondrial affinity for ADP, where an increased matrix volume was associated with reduced ADP affinity (56), but this mechanism would rather sensitize mitochondria to mPT than increase CRC. The expanded matrix volume may, however, hold more P_i and reduce some physicochemical restraints in the protein dense matrix to facilitate calcium complex formation.

Mild Uncoupling and Calcium Retention—Mild uncoupling of mitochondria, *i.e.* slightly reducing the proton motive force and $\Delta\Psi_m$ by increasing H^+ conductance by, e.g. protonophores, has been suggested to be a beneficial strategy against mitochondrial-mediated cell damage via decreased ROS generation and limitation of mitochondrial calcium uptake (57, 58). Diazoxide has been shown to concentration-dependently (IC_{50} 65 μM) decrease the rate and magnitude of calcium uptake in isolated heart mitochondria (58). However, the authors further reported that diazoxide activated mitochondrial release of calcium, a process that was prevented by cyclosporin A. This indicates that diazoxide at high concentrations actually sensitized the mitochondria toward mPT and the associated calcium

release (which we also demonstrate in the present study) rather than afforded protection against mPT. It is likely that these effects of diazoxide are related to unspecific respiratory inhibition and depolarization at high concentrations rather than specific activity of mitoK_{ATP}. A hampered mitochondrial function caused by high dosing of diazoxide or other pharmacological agents secondarily reducing calcium uptake and retention are likely not compatible with the high energy demand of neuronal cells, in particular during pathological situations of increased metabolic stress and cytoplasmic calcium overload (59). Mimicking K⁺ channel activity with valinomycin demonstrated an opposite effect to diazoxide (and CCCP) on CRC in our hands. We suggest that if mild uncoupling is beneficial, increased K⁺ conductance rather than increased H⁺ conductance may be a safer approach to reduce ROS as it would not pose the threat of sensitizing mitochondria to mPT during calcium accumulation.

MitoK_{ATP}—Several lines of evidence indicate the existence of mitoK_{ATP} and a prominent role of these channels in the preconditioning effect of cardiac and cerebral ischemia (7, 8, 12). Several authors have, however, failed to detect the existence of mitoK_{ATP} in isolated heart and brain mitochondria (32, 33), and the specificity of diazoxide and 5-hydroxydecanoate has been questioned (43, 60). Lack of specific effects of diazoxide could indicate that mitoK_{ATP} are not present in mitochondria and that the pharmacological effects of diazoxide and other putative ligands of this channel are caused by interaction with other pharmacological or unspecific targets. In line with this is the suggestion that ischemic and pharmacological preconditioning can induce protection through nonspecific effects on mitochondrial function including respiratory chain inhibition and ROS release from the mitochondria (21). Another alternative is that the channel is inactivated during isolation of mitochondria or that a cofactor needs to be present, in which cases the variable findings can depend on minor differences in mitochondrial isolation procedures. Although the existence of mitoK_{ATP} may be controversial, electrophoretic K⁺ flux in mitochondria is well established (26, 46, 61), and the concept of altered K⁺ flux of the inner mitochondrial membrane in preconditioning remains thrilling. A Kv1.3 and a Ca²⁺-activated K⁺ channel are other candidates for mitochondrial K⁺ channels relevant for preconditioning, and ligands of the latter demonstrate a similar cardioprotective effect as ligands of mitoK_{ATP} (60, 62–64).

Clinical Perspective—Inhibition of cyclophilin D, thereby reducing the sensitivity to mPT, is a potential strategy for neuroprotection in acute and chronic neurodegenerative disease as well as for cardioprotection in ischemia-reperfusion injury. The cyclophilin D inhibitor cyclosporin A has displayed promising results in a human clinical trial of myocardial reperfusion injury (65) and human clinical trials in traumatic brain injury (66–68). The findings of the present study indicate that activation of mitochondrial K⁺ channels could provide an additional means of improving mitochondrial resistance to calcium overload and activation of mPT through an enhanced matrix volume. Augmenting the brain mitochondrial ability to retain calcium by matrix volume modulation may, thus, be an end-effector of

preconditioning and ischemic tolerance and could constitute a novel target for therapeutic intervention.

Acknowledgments—We are grateful to David Nicholls, Andrew Halestrap, and Carsten Ruscher for methodological input, to Tadeusz Wieloch for support, and to Fredrik Leeb-Lundberg for use of scintillation counter. *Debio-025 (D-MeAla³EtVal⁴-cyclosporin)* was kindly provided by *Debiopharm S.A.*

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