Cd\(^{2+}\) -induced swelling-contraction dynamics in isolated kidney cortex mitochondria: role of Ca\(^{2+}\) uniporter, K\(^{+}\) cycling, and protonmotive force

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Lee, Wing-Kee, Malte Spielmann, Ulrich Bork, and Frank Thévenod. Cd\(^{2+}\) -induced swelling-contraction dynamics in isolated kidney cortex mitochondria: role of Ca\(^{2+}\) uniporter, K\(^{+}\) cycling, and protonmotive force. Am J Physiol Cell Physiol 289: C656 –C664, 2005. First published April 20, 2005; doi:10.1152/ajpcell.00049.2005.—The nephrototoxic metal Cd\(^{2+}\) causes mitochondrial damage and apoptosis of kidney proximal tubule cells. A K\(^{+}\) cycle involving a K\(^{+}\) uniporter and a K\(^{-}\)/H\(^{+}\) exchanger in the inner mitochondrial membrane (IMM) is thought to contribute to the maintenance of the structural and functional integrity of mitochondria. In the present study, we have investigated the effect of Cd\(^{2+}\) on K\(^{+}\) cycling in rat kidney cortex mitochondria. Cd\(^{2+}\) (EC\(_{50}\) ~19 \(\mu\)M) induced swelling of nonenergized mitochondria suspended in isotonic salt solutions according to the sequence KCl = NaCl > LiCl >> choline chloride. Cd\(^{2+}\)-induced swelling of energized mitochondria had a similar EC\(_{50}\) value and showed the same cation dependence but was followed by a spontaneous contraction. Mitochondrial Ca\(^{2+}\) uniporter (MCU) blockers, but not permeability transition pore inhibitors, abolished swelling, suggesting the need for Ca\(^{2+}\) influx through the MCU for swelling to occur. Complete loss of mitochondrial membrane potential (\(\Delta\psi_{m}\)) induced by K\(^{-}\) influx did not prevent contraction, but addition of the K\(^{-}\)/H\(^{+}\) exchanger blocker, quinine (1 mM), or the electroneutral protonophore nigericin (0.4 \(\mu\)M), abolished swelling, suggesting the mitochondrial pH gradient (\(\Delta\psi_{m}\)) driving contraction. Accordingly, a quinine-sensitive partial dissipation of \(\Delta\psi_{m}\) was coincident with the swelling-contraction phase. The data indicate that Cd\(^{2+}\) enters the matrix through the MCU to activate a K\(^{+}\) cycle. Initial K\(^{+}\) load via a Cd\(^{2+}\)-activated K\(^{+}\) uniporter in the IMM causes osmotic swelling and breakdown of \(\Delta\psi_{m}\) and triggers quinine-sensitive K\(^{-}\)/H\(^{+}\) exchange and contraction. Thus Cd\(^{2+}\)-induced activation of a K\(^{+}\) cycle contributes to the dissipation of the mitochondrial protonmotive force.

bongkrekic acid; cyclosporin A; lanthanum; Ru360; ruthenium red

Cadmium has no known physiological function but shares some toxic properties with other heavy metals (16, 57, 58). A focal point for chronic Cd\(^{2+}\) toxicity is the proximal tubule (PT), particularly in its S1 segment (49, 57). Renal dysfunction in the form of proteinuria, aminoaciduria, glucosuria, and phosphaturia has been demonstrated in people occupationally exposed to Cd\(^{2+}\) (for review, see Refs. 31, 55); these symptoms resemble the de Toni-Debré-Fanconi syndrome and may affect up to 7% of the general population (27, 30).

When free Cd\(^{2+}\) reaches the cytosol of PT cells, it induces the formation of reactive oxygen species (ROS) and subsequent damage to critical organelles (49, 57). If ROS-mediated stress events are not sufficiently balanced by protective and repair processes, PT cells affected by Cd\(^{2+}\) are induced to undergo cell death via apoptosis or necrosis in vivo (34, 48) and in vitro (50, 51). After a toxic stimulus such as Cd\(^{2+}\), a number of proapoptotic proteins are liberated from the intermembrane space (IMS) of mitochondria into the surrounding cytosol, and these proteins activate downstream pathways (41, 49). Factors that are normally sequestered in the mitochondria but are released upon apoptotic signals include cytochrome c (35), apoptosis-inducing factor (AIF) (47), and Smac/Diablo (12, 52), as well as a number of procaspases, which appear to be key elements in the cascade of biochemical events leading to apoptosis.

It has been hypothesized that temporary or permanent alterations of the integrity of the mitochondrial inner and/or outer membrane induce the release of these proapoptotic proteins (6, 32, 36). Various mechanisms have been implicated in alterations of the mitochondrial membrane permeability and release of cytochrome c that either require Ca\(^{2+}\) or are Ca\(^{2+}\) independent. The latter mechanism may involve members of the Bcl-2 protein family in the release of cytochrome c but is not necessarily associated with changes in mitochondrial volume (44, 45, 53). Alternatively, the opening of a permeability transition pore (PTP) is activated by Ca\(^{2+}\), resulting in swelling and rupture of the outer membrane followed by the release of proapoptotic mitochondrial proteins (9, 22). Characteristic properties of PTP are its activation by Ca\(^{2+}\) and inhibition by cyclosporin A (CsA) (10). However, there is additional evidence from studies in intact cells and isolated mitochondria that changes of mitochondrial membrane permeability and/or release of cytochrome c may also occur by processes that are independent of these canonical mechanisms (2, 3, 19, 20, 23, 40). We recently demonstrated that Cd\(^{2+}\) induces swelling and release of cytochrome c from energized kidney cortex mitochondria suspended in mannitol-sucrose-HEPES (MSH) buffer independently of PTP opening (33). Rather, Cd\(^{2+}\) entered the matrix space through the mitochondrial Ca\(^{2+}\) uniporter (MCU) to activate aquaporin H\(_{2}\)O channels and H\(_{2}\)O influx resulted in osmotic swelling and release of cytochrome c (33).

In mitochondria suspended in KCl buffer, a K\(^{+}\) cycle has been proposed to contribute to the maintenance of the structural and functional integrity of mitochondria. It involves K\(^{+}\) influx by activation of K\(^{+}\) uniporters or channels in the inner mitochondrial membrane (IMM) and K\(^{-}\)/H\(^{+}\) exchange, which regulates physiological mitochondrial volume homeostasis (17). Mitochondria possess several K\(^{+}\) channels or uniporters in the IMM, such as the K\(^{-}\)- and Na\(^{+}\)-permeable mitochondrial ATP-sensitive channel (K\(_{ATP}\) channel) (11, 25) or the...
Ca\(^{2+}\)-stimulated, voltage-dependent, and exclusively K\(^+\)-selective channel (KC\(_a\) channel) (46, 56). It also has been suggested that activation of both channels may contribute to cytotoxicity against the mitochondrial death pathway induced by hypoxia in cardiac cells (1, 56). However, Eliseev and colleagues (13, 14) described CsA-independent swelling and cytotoxicity release in mitochondria isolated from etoposide-treated HL-60 cells and suspended in KCl buffer that were caused by increased K\(^+\) influx through a K\(^+\) uniporter.

In the present study, we have investigated the effect of Cd\(^{2+}\) on rat kidney cortex mitochondria suspended in isotonic KCl buffer. Cd\(^{2+}\) (5–50 \(\mu\)M) induced K\(^+\)-dependent osmotic swelling of nonenergized or energized kidney cortex mitochondria by a process involving a K\(^+\) uniporter and the MCU, but not the PTP. In energized mitochondria only, Cd\(^{2+}\) induces a biphasic mitochondrial osmotic swelling-contraction response. Evidence is provided that the underlying process is Cd\(^{2+}\)-induced K\(^+\) cycling involving K\(^+\) influx through the K\(^+\) uniporter and K\(^+\) efflux through a quinine-sensitive K\(^+\)/H\(^+\) antiporter, which is driven by the mitochondrial pH gradient (\(\Delta p\text{H}_{\text{m}}\)).

**EXPERIMENTAL PROCEDURES**

**Materials**

CdCl\(_2\) was obtained from Merck (Darmstadt, Germany). Alamethicin, bongkrekic acid (BKA), carbonyl cyanide m-chlorophenylhydrazone (CCCP), CsA, dioxazine, 5-hydroxydecanoate (5-HD), nigericin, quinine hydrochloride, rhodamine 123\(^+\) (Rh123\(^+\)), rotenone, and ruthenium red (RR) were all purchased from Sigma (St. Louis, MO). KCl and sodium succinate were prepared as solutions of 100 mM. Cd\(^{2+}\) was prepared as a solution of 10 mM. Mitochondria were energized with 10 mM sodium succinate (titrated to pH 7), and nigericin (0.4 \(\mu\)M). Data were captured and converted using DU-WinConnection Suite software and analyzed using Microsoft Excel and SigmaPlot 8.0 (SPSS, Chicago, IL). Rates of swelling (\(\Delta\text{absorbance}_{440 \text{nm}}/\text{min}\)) were calculated on the basis of the initial linear portion of mitochondrial absorbance curves and the rates of contraction (\(\Delta\text{absorbance}_{440 \text{nm}}/\text{min}\)) from the initial portion of the contraction phase. Swelling curves were normalized to maximal mitochondrial swelling. To determine maximal swelling, mitochondria were suspended in KCl buffer and the maximal absorbance changes induced by the monovalent cation ionophore alamethacin (12.5 \(\mu\)g per mg/ml protein) were defined as 100% swelling. Alamethicin-induced absorbance changes amounted to 53 ± 1.4% of initial absorbance. To simplify calculations, maximal swelling was assumed to be 50% of initial absorbance. Normalized values were obtained from the formula \(1 - [\Delta\text{absorbance}_{440\text{nm}}/\text{initial absorbance}]\) × 100 and expressed as %swelling, which means swelling increase relative to the alamethacin-induced maximal signal.

**Determination of mitochondrial membrane potential**. As an indication of the changes of mitochondrial membrane potential (\(\Delta \text{V}_{\text{m}}\)), the ability of the mitochondria to sequester the cationic and lipophilic green fluorescent dye Rh123\(^+\) (\(\lambda_{\text{excitation}} = 454 \text{nm}, \lambda_{\text{emission}} = 550 \text{nm}\)) was monitored using an LS50B luminescence spectrophotometer (PerkinElmer, Wellesley, MA) and the data were captured using a FL Data Manager program (PerkinElmer). In energized mitochondria, Rh123\(^+\) accumulation into the mitochondrial matrix is driven by the inside negative \(\Delta \text{V}_{\text{m}}\) of the IMM and the fluorescence becomes quenched. Upon depolarization of the IMM, the dye is released into the surrounding medium, and Rh123\(^+\) quenching decreases, causing an increase in fluorescence intensity (15). Mitochondria (0.3 mg/ml) were added to 3 ml of a buffer (in mM: 110 mannitol, 60 Tris, and 60 KCl; pH 7.4, with HCl) containing 2.6 \(\mu\)g Rh123\(^+\) and 5 mM MgCl\(_2\).

The mitochondria were energized with 10 mM sodium succinate and 1 \(\mu\)M rotenone. The protonophore CCCP (0.3 \(\mu\)M) was used as a positive control to dissipate \(\Delta \text{V}_{\text{m}}\). In preliminary experiments, nonspecific signals and/or false-positive results due to interference of the tested compounds with Rh123\(^+\) were excluded.

**Measurement of \(\Delta \text{pH}_{\text{m}}\)**. The protocol described by Kapus et al. (28) was used with slight modifications. Mitochondria (40–50 mg/ml) were preloaded with 10 \(\mu\)M BCECF-AM for 30 min at room temperature. BCECF-loaded mitochondria were centrifuged at 10,000 g for 5 min at 4°C, and the supernatant was discarded. The pellet was resuspended in mitochondria isolation buffer (in mM: 210 mannitol, 70 sucrose, and 5 HEPES, pH 7.4, with Tris) and further diluted 20-fold with the same buffer. Mitochondria were centrifuged again as described above. Supernatants were discarded and pellets were resuspended to the original starting concentration with mitochondria isolation buffer. Mitochondria (1 mg/ml) were added to 3 ml of experimental buffer (140 mM KCl, 10 mM MOPS, 2.5 \(\mu\)g/ml oligomycin, 1 \(\mu\)M rotenone, pH 7, with KOH). BCECF fluorescence intensity was measured at \(\lambda_{\text{excitation}} = 503 \text{nm}\) and \(\lambda_{\text{emission}} = 528 \text{nm}\) on a PerkinElmer LS50B luminescence spectrophotometer, and data were captured with the FL Data Manager program. Mitochondria were energized with 10 mM sodium succinate (titrated to pH 7), and nigericin (0.4 \(\mu\)M) and CCCP (1 \(\mu\)M) were used as a positive control to dissipate \(\Delta \text{pH}_{\text{m}}\). In preliminary experiments, nonspecific signals and/or false-positive results due to interference of the tested compounds with BCECF were excluded.

**Statistical Analyses**

All experiments were repeated at least three times with different preparations of mitochondria. Representative data or means ± SE are shown. Statistical analysis using an unpaired Student’s t-test was performed with the SigmaPlot 8.0 spreadsheet software program. For more than two groups, statistical differences were compared using one-way ANOVA assuming equality of variance with Levene’s test.
and Tukey’s post hoc test for pairwise comparison. Statistical analysis was performed with the SPSS 11.0 software program. Results with \( P \leq 0.05 \) were considered to be statistically significant. To obtain EC_{50} values, dose-response curves of Cd\(^{2+}\)-induced mitochondrial swelling were fitted using the SigmaPlot 8.0 spreadsheet software program assuming a sigmoidal dose-response curve (variable slope). Dose-response curves of Cd\(^{2+}\) effects on mitochondrial membrane potential (\( \Delta \Psi_m \)) were curve fitted assuming a hyperbolic function according to the equation for one-site saturation plus nonspecific binding using SigmaPlot 8.0, and EC_{50} values were determined.

RESULTS

**Cd\(^{2+}\) Induces Swelling of Nonenergized Mitochondria in KCl Buffer**

Addition of Cd\(^{2+}\) (5–50 \( \mu \)M) induced swelling of nonenergized rat kidney cortex mitochondria suspended in KCl buffer (Fig. 1A). The kinetics of swelling during a period of up to 12 min had a characteristic pattern showing an initial swelling phase that reached a plateau within 1–2 min. The initial rate of swelling during the first minute increased as a function of the Cd\(^{2+}\) concentration and saturated at \( \sim 100 \) \( \mu \)M Cd\(^{2+}\) (EC_{50} = 18.8 \pm 2.4 \( \mu \)M; \( n = 4 \)). The magnitude of the plateau showed a similar dependence on the Cd\(^{2+}\) concentration (data not shown). When K\(^+\) was replaced by other monovalent cations, the initial rates of swelling induced by 10 or 50 \( \mu \)M Cd\(^{2+}\) were reduced according to the sequence K\(^+\) = Na\(^+\) > Li\(^+\) \( \gg\) choline\(^+\) independently of the Cd\(^{2+}\) concentration tested (Fig. 1B). Replacement of K\(^+\) by the bulky cation choline\(^+\) decreased swelling by \( \sim 70\% \), which indicates that Cd\(^{2+}\) activates K\(^+\)- and Na\(^+\)-permeable influx pathways, which cause osmotic swelling of the mitochondrial matrix.

In an attempt to identify the underlying K\(^+\) channel, we tested the specific inhibitor of the mitochondrial K\(_{ATP}\) channel, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (26), and the activator diazoxide up to 50 \( \mu \)M (18). Both drugs had no effect on Cd\(^{2+}\)-induced swelling in KCl buffer (data not shown), suggesting that Cd\(^{2+}\) does not activate a mitochondrial K\(_{ATP}\) channel.

**Cd\(^{2+}\)-Induced Swelling of Energized Mitochondria in KCl Buffer Is Biphasic**

Cd\(^ {2+}\) also caused swelling of energized rat kidney cortex mitochondria suspended in KCl buffer, but the kinetics of swelling were more complex (Fig. 2). Swelling occurred more rapidly, but the amplitude of swelling was smaller. More important, the initial phase of swelling was immediately followed by a phase of mitochondrial contraction to control levels at concentrations >2 \( \mu \)M Cd\(^{2+}\) (Fig. 2). The initial rate of swelling during the first minute was also a function of the Cd\(^{2+}\) concentration and saturated at 20 \( \mu \)M Cd\(^{2+}\) (EC_{50} = 8.4 \pm 0.7 \( \mu \)M; \( n = 8 \)). Once mitochondrial contraction had reached control absorbance values, it was maintained for at least 6–8 min after addition of Cd\(^{2+}\) (see, e.g., Fig. 4A). Contraction was observed in Na\(^{+}\)-free buffers as well but was abolished in the presence of choline\(^+\) (data not shown). These data indicate that the mechanism underlying the initial rate of swelling is K\(^+\) dependent but does not require energization of mitochondria, whereas the contraction phase is also K\(^+\) dependent but requires the generation of \( \Delta \Psi_m \) and/or \( \Delta \Psi_m \) to operate.

**Cd\(^{2+}\)-Induced Mitochondrial Swelling in KCl Buffer Is PTP Independent but MCU Dependent**

We have previously shown in kidney cortex mitochondria suspended in MSH buffer that swelling induced by Cd\(^{2+}\) is independent of opening of the PTP but requires Cd\(^{2+}\) influx into the mitochondrial matrix through the MCU to occur (33). CsA (1 \( \mu \)M), a potent inhibitor of the PTP (10) or 5 \( \mu \)M BKA, another drug that blocks the PTP by binding to the adenine nucleotide translocator (ANT) (21), did not significantly affect initial rates of swelling of nonenergized or energized mitochondria induced by 20–50 \( \mu \)M Cd\(^{2+}\) (Table 1; see also Ref. 33), which makes the involvement of the PTP in the process of swelling of mitochondria suspended in KCl buffer very unlikely.

In contrast, inhibitors of the MCU prevented swelling in KCl buffer. Table 1 shows that the noncompetitive MCU inhibitors, RR and its active component Ru360, as well as the competitive inhibitor La\(^{3+}\) (for review, see Refs. 5, 59), strongly inhibited
Cadmium and Mitochondrial Potassium Cycle

**Cd\(^{2+}\)**-induced swelling of nonenergized or energized kidney cortex mitochondria suspended in KCl buffer. Swelling induced by 10 \(\mu\)M Cd\(^{2+}\) was abolished by 100 nM Ru360 (Table 1). RR inhibited osmotic swelling of energized mitochondria induced by 50 \(\mu\)M Cd\(^{2+}\) with an IC\(_{50}\) of 27.7 ± 7.2 (\(n = 4\)). Overall, the data indicate that Cd\(^{2+}\) transport into the mitochondrial matrix through the MCU is necessary to induce K\(^{+}\) permeability from the matrix side, with the K\(^{+}\) influx resulting in mitochondrial swelling.

**Cd\(^{2+}\)** Influx Through the MCU Induces Rapid Dissipation of \(\Delta\Psi_m\)

Figure 3A shows that addition of Cd\(^{2+}\) results in dissipation of \(\Delta\Psi_m\) in energized kidney cortex mitochondria with an EC\(_{50}\) of ~11.5 \(\mu\)M Cd\(^{2+}\) (Fig. 3A, inset). A rapid breakdown of \(\Delta\Psi_m\) was observed within 1 min at Cd\(^{2+}\) concentrations >5 \(\mu\)M. Consequently, breakdown of \(\Delta\Psi_m\) occurred at time points when mitochondrial contraction was still operative (see Figs. 2 and 4A), which indicates that \(\Delta\Psi_m\) is not necessary for mitochondrial contraction to occur. Ru360 also reduced Cd\(^{2+}\)-induced dissipation of \(\Delta\Psi_m\) by ~50% (Fig. 3B), which suggests that Cd\(^{2+}\)-induced electrogenic K\(^{+}\) influx through K\(^{+}\) permeability is responsible for dissipation of \(\Delta\Psi_m\).

**Cd\(^{2+}\)**-Induced Mitochondrial Contraction Phase Requires a \(\Delta\pi_m\)

Whereas mitochondrial contraction of energized mitochondria was still observed while \(\Delta\Psi_m\) was completely dissipated (Fig. 3A), dissipation of \(\Delta\pi_m\) by the addition of the electroneutral K\(^{+}\)/H\(^{+}\) exchanger nigericin (0.4 \(\mu\)M) at the peak of mitochondrial swelling (when K\(^{+}\) in buffer and matrix space are at equilibrium) abolished mitochondrial contraction induced by 10 \(\mu\)M Cd\(^{2+}\) (Fig. 4, A and B). The electrogenic ionophore CCCP (0.3 \(\mu\)M) had the same effect (data not shown), but it cannot be used as a valid tool to determine the role of \(\Delta\pi_m\), because it simultaneously affected \(\Delta\pi_m\) and \(\Delta\Psi_m\) (Fig. 3A). These experiments suggest that a pH gradient across the IMM is required for mitochondrial contraction to occur.

**Activation of the Mitochondrial K\(^{+}\)/H\(^{+}\) Exchanger Mediates the Contraction Phase of Mitochondrial Swelling**

As shown in Fig. 5, A and B, the contraction phase of osmotic swelling induced by 10 \(\mu\)M Cd\(^{2+}\) was abolished when an inhibitor of the K\(^{+}\)/H\(^{+}\) exchanger of the IMM (quinine; 1 mM) (38) was added at the peak of mitochondrial swelling. Moreover, the addition of quinine to control mitochondria also enhanced swelling, suggesting that basal activity of the mitochondrial K\(^{+}\)/H\(^{+}\) exchanger contributes to the stability of energized kidney mitochondria suspended in KCl buffer.

Next, we measured \(\Delta\pi_m\) with BCECF. When mitochondria were energized with 10 mM succinate, BCECF fluorescence increased, reflecting that alkalinization of the matrix space and addition of the protonophores CCCP (1 \(\mu\)M) and nigericin (0.4 \(\mu\)M) abolished the pH gradient generated in energized mito-

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**Table 1. Effect of PTP and MCU inhibitors on Cd\(^{2+}\)**-induced swelling of mitochondria suspended in KCl buffer

<table>
<thead>
<tr>
<th>Cd(^{2+}) Concentration ± Inhibitor</th>
<th>Energized Mitochondria, (\Delta\text{absorban}c_{540 \text{nm}}\text{min}^{-1} \times 10^3)</th>
<th>Nonenergized Mitochondria, (\Delta\text{absorban}c_{540 \text{nm}}\text{min}^{-1} \times 10^3)</th>
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</thead>
<tbody>
<tr>
<td>PTP inhibitors</td>
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<td></td>
</tr>
<tr>
<td>50 (\mu)M Cd(^{2+})</td>
<td>199.2 ± 17.8</td>
<td>148.4 ± 20.7</td>
</tr>
<tr>
<td>50 (\mu)M Cd(^{2+}) + 1 (\mu)M CsA</td>
<td>189.8 ± 22.4</td>
<td>137.8 ± 46.4</td>
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<tr>
<td>50 (\mu)M Cd(^{2+}) + 5 (\mu)M BKA</td>
<td>230.0 ± 29.5</td>
<td>149.4 ± 15.6</td>
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<tr>
<td>MCU inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 (\mu)M Cd(^{2+})</td>
<td>114.9 ± 3.9</td>
<td>95.6 ± 3.0</td>
</tr>
<tr>
<td>10 (\mu)M Cd(^{2+}) + 0.2 (\mu)M RR</td>
<td>21.9 ± 5.8†</td>
<td>29.5 ± 0.5†</td>
</tr>
<tr>
<td>10 (\mu)M Cd(^{2+}) + 0.1 (\mu)M Ru360</td>
<td>16.9 ± 3.3†</td>
<td>134.5 ± 27.4</td>
</tr>
<tr>
<td>10 (\mu)M Cd(^{2+}) + 5 (\mu)M La(^{3+})</td>
<td>209.3 ± 17.9</td>
<td>82.2 ± 4.0†</td>
</tr>
<tr>
<td>50 (\mu)M Cd(^{2+})</td>
<td>8.65 ± 18.9†</td>
<td>50.6 ± 10.6†</td>
</tr>
<tr>
<td>50 (\mu)M Cd(^{2+}) + 0.2 (\mu)M RR</td>
<td>18.4 ± 10.9†</td>
<td></td>
</tr>
<tr>
<td>50 (\mu)M Cd(^{2+}) + 0.1 (\mu)M Ru360</td>
<td>8.65 ± 18.9†</td>
<td></td>
</tr>
<tr>
<td>50 (\mu)M Cd(^{2+}) + 5 (\mu)M La(^{3+})</td>
<td>50.6 ± 10.6†</td>
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</tbody>
</table>

Values are means ± SE of 3–7 experiments. Swelling of isolated nonenergized and energized mitochondria was induced by 10–50 \(\mu\)M Cd\(^{2+}\) in the absence or presence of inhibitors. Rates of absorbance changes per minute after addition of Cd\(^{2+}\) were analyzed. Inhibitors of the permeability transition pore (PTP), cyclosporin A (CsA), and bongkrekic acid (BKA), and mitochondrial Cd\(^{2+}\) unipporter (MCU) inhibitors, ruthenium red (RR), Ru360, and La\(^{3+}\), were present in the buffer throughout the experiment. *\(P < 0.05\); †\(P < 0.01\). Statistical analysis was performed using Student’s unpaired t-test (nonenergized mitochondria) and one-way ANOVA and post hoc Tukey’s test for multiple comparisons (energized mitochondria) to compare Cd\(^{2+}\)-only controls with Cd\(^{2+}\) in the presence of inhibitor.

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The addition of 10 μM Cd²⁺ induced a steady decrease of pH_m during a period of 1–2 min until a new steady state well above the level of nonenergized mitochondria was reached (51.4 ± 5.9% of total F; n = 5) that could be abolished by the addition of CCCP and nigericin. The rates of both dissipation of pH_m and equilibrium pH_m after 10 μM Cd²⁺ were significantly lower than those obtained after dissipation of pH_m with CCCP and nigericin (data not shown). Furthermore, quinine also decreased the initial dissipation rate of pH_m observed after addition of 10 μM Cd²⁺ (Fig. 5C), which indicates that activation of the mitochondrial K⁺/H⁺ exchanger contributes to the dissipation of pH_m.

DISCUSSION

How Does Cd²⁺ Affect PTP-Independent Mitochondrial Swelling?

CsA and BKA did not inhibit Cd²⁺-induced swelling of kidney cortex mitochondria suspended in KCl buffer (Table 1), which confirms the findings of our previous study (33). This demonstrates that Cd²⁺-induced swelling of kidney cortex mitochondria is not involved in the activation of the PTP. Other studies also have reported mitochondrial swelling and cytochrome c release in isolated mitochondria induced by stimuli such as Hg²⁺ (13, 24), etoposide (13, 14), valinomycin (19), or long-chain fatty acids (43) that were not inhibited by CsA, suggesting that the classical PTP was not involved in

Fig. 3. Effect of Cd²⁺ on mitochondrial membrane potential (ΔΨ_m). A: inner mitochondrial membrane (IMM) potential was monitored using rhodamine 123 (Rh123). Isolated mitochondria (mito) were added and energized with succinate and rotenone. After stabilization of the fluorescence signal (in relative fluorescence units, rfu), 1–50 μM Cd²⁺ was added. Fluorescence changes were monitored for 6.5 min before uncoupling by carbonyl cyanide m-chlorophenylhydrazone (CCCP; 0.3 μM). A: trace representative of 4–10 experiments (100% = 135.0 ± 2.2 rfu). Inset, dose-response relationship for Cd²⁺-induced loss of ΔΨ_m, plotted as ΔF/min assuming a curve fit for one-site saturation and nonspecific binding. EC₅₀ value ± SE was derived from five individual mitochondrial preparations. B: Ru360 (10 μM) was added to the experimental buffer before addition of isolated mitochondria. The initial rates of Rh123 fluorescence changes (ΔF/min) induced by Cd²⁺ were analyzed in the absence and in the presence of the mitochondrial Ca²⁺ uniporter (MCU) inhibitor Ru360. Statistical analysis using Student’s unpaired t-test was performed to compare Ru360 and Cd²⁺-treated with Cd²⁺ only-treated mitochondria. Bar graph shows means ± SE of seven experiments. **P < 0.0125.

Fig. 4. Effects of nigericin on Cd²⁺-induced contraction of mitochondria suspended in KCl buffer. A: mitochondrial swelling experiments were conducted as described in the Fig. 2 legend. Swelling of energized mitochondria was induced by 10 μM Cd²⁺. Where indicated, 0.4 μM nigericin was added to mitochondria at the peak of swelling induced by 10 μM Cd²⁺ (arrow), and swelling was monitored for a further 6 min. B: absorbance changes (Δabsorbance540 nm/min) during the initial phase of contraction were analyzed for each condition. Statistical analysis using one-way ANOVA with Tukey’s post hoc test was performed to compare treated mitochondria with control as well as Cd²⁺-nigericin-CCCP treated with Cd²⁺ only-treated mitochondria. Bar graph shows means ± SE of three experiments. *P < 0.05. **P < 0.01.
these processes. For instance, Eliseev and colleagues (13, 14) described CsA-independent swelling and cytochrome c release in mitochondria isolated from etoposide-treated HL-60 cells that were caused by increased K\(^+\) influx through the K\(^+\) uniporter. These effects also have been mimicked with the electrogenic K\(^+\) ionophore valinomycin (19).

K\(^+\)-dependent swelling induced by Cd\(^{2+}\) was observed in nonenergized and energized rat kidney cortex mitochondria (Figs. 1 and 2). Whereas K\(^+\) influx into nonenergized mitochondria is driven by the concentration gradient between the medium and the matrix space, in energized mitochondria, $\Delta\Psi_m$ in addition drives K\(^+\) influx. Indeed, $\Delta\Psi_m$ may play a significant role in the initiation of K\(^+\) influx, because swelling occurred more rapidly in energized mitochondria than in non-energized ones (compare Figs. 1 and 2). This may also have an effect on the EC\(_{50}\) values used to describe the effectiveness of Cd\(^{2+}\) (~19 $\mu$M for nonenergized and ~8 $\mu$M for energized mitochondria). The different values may appear to suggest that Cd\(^{2+}\) is more effective in energized mitochondria, which may be misleading. However, these differences likely reflect differences in the driving forces for K\(^+\) (and Cd\(^{2+}\)), because the measured indirect parameter “swelling” reflects Cd\(^{2+}\)-dependent K\(^+\) influx rather than direct binding affinity of Cd\(^{2+}\) to the Ca\(^{2+}\) uniporter. In contrast, the amplitude of swelling was smaller in energized mitochondria, which indicates that the electrochemical gradient across the IMM may counteract the process of swelling.

We also found that Cd\(^{2+}\)-activated, K\(^+\)-mediated swelling was abolished by inhibition of the MCU with RR or Ru360 (Table 1). This suggests that Cd\(^{2+}\) must enter the matrix space to induce K\(^+\) influx. Swelling could occur by activation of K\(_{Ca}\) conductance in the IMM. Halestrap et al. (23) previously showed that Ca\(^{2+}\)-activated K\(^+\) influx into energized liver mitochondria is prevented by RR and concluded that Ca\(^{2+}\) must enter the mitochondria to activate Ca\(^{2+}\)-sensitive, K\(^+\)-dependent swelling. Moreover, in patch-clamp experiments, Siemen et al. (46) found the Ca\(^{2+}\) binding site of the mitochondrial K\(^+\)-selective K\(_{Ca}\) channel to be on the matrix side. In our hands, Cd\(^{2+}\)-induced mitochondrial swelling was not K\(^+\) selective and followed a selectivity sequence of K\(^+\) = Na\(^+\) > Li\(^+\) > choline\(^+\) in nonenergized mitochondria (Fig. 1). Our data are also similar to those associated with swelling induced by long-chain fatty acids, which does not show any K\(^+\) selectivity (42). In HL-60 cells, K\(^+\)-dependent mitochondrial swelling and cytochrome c release are associated with the activation of a K\(_{ATP}\) channel (14). However, neither the specific inhibitor of the mitochondrial K\(_{ATP}\) channel, 5-HD (26), nor the specific opener of K\(_{ATP}\) channels, diazoxide (18), affected Cd\(^{2+}\)-induced mitochondrial swelling (data not shown). This suggests that a mitochondrial K\(_{ATP}\) channel does not contribute to K\(^+\)-dependent swelling.

Fig. 5. Effect of quinine on Cd\(^{2+}\)-induced contraction of mitochondria suspended in KCl buffer (A and B) and dissipation of ($\Delta$P$_{H\text{m}}$) (C). A: swelling experiments were conducted as described for Fig. 2. Quinine (1 mM) was added to mitochondria at the peak of swelling induced by 10 $\mu$M Cd\(^{2+}\) (arrow). B: absorbance rates ($\Delta$absorbance$_{600}$/min) during the initial phase of contraction were analyzed for 10 $\mu$M Cd\(^{2+}\) ± 1 mM quinine in KCl buffer and compared with absorbance changes of controls ± 1 mM quinine. Statistical analysis using one-way ANOVA with Tukey’s post hoc test was performed to compare Cd\(^{2+}\) ± quinine-treated mitochondria with controls as well as to compare Cd\(^{2+}\) ± quinine with Cd\(^{2+}\) only. Bar graph shows means ± SE of five experiments. *$P <$ 0.05; **$P <$ 0.01. C: experiments were conducted as described. Cd\(^{2+}\) (10 $\mu$M) was added to energized mitochondria in the absence and in the presence of 1 mM quinine, and BCECF fluorescence was monitored for 4 min before breakdown of $\Delta$P$_{H\text{m}}$ by 0.4 $\mu$M nigericin and 1 $\mu$M CCCP. Traces are representative of four individual experiments.
We could also exclude with certainty the alternative possibility of Cd$^{2+}$ cycling through the MCU and a Na$^+$/Ca$^{2+}$ antiporter being responsible for swelling. Apart from the fact that the osmotic load generated by micromolar Cd$^{2+}$ concentrations is too low to account for swelling and that swelling is clearly K$^+$ dependent (Fig. 1), 20–50 μM concentrations of Sr$^{2+}$, which permeate the MCU (29) and the Na$^+$/Ca$^{2+}$ antiporter (37) equally as well as Ca$^{2+}$, did not induce any swelling of mitochondria suspended in KCl buffer (data not shown).

The observation that the replacement of K$^+$ by the bulky cation choline$^+$ did not completely abolish Cd$^{2+}$-induced swelling (Fig. 1B) indicates that several IMM pathways must contribute to Cd$^{2+}$-induced mitochondrial swelling. It is noteworthy that Cd$^{2+}$-induced swelling with choline$^+$ had approximately the same magnitude (∼25% of swelling in KCl buffer) because Cd$^{2+}$ induced swelling in MSH buffer, where H$_2$O flow through Cd$^{2+}$-activated aquaporin-8 H$_2$O channels accounts for osmotic swelling (33). Thus it is likely that Cd$^{2+}$ activates one or several more or less K$^+$-selective channels or uniporters, such as a nonselective K$^+$ channel (5), which results in K$^+$ influx that is driven by the electrochemical potential across the IMM. Because Cd$^{2+}$ also activates H$_2$O channels (33), H$_2$O follows the osmotic K$^+$ load into the matrix space.

The extent of swelling induced by Cd$^{2+}$ was quite different in energized and nonenergized mitochondria (compare Figs. 1A and 2). Hence it would have been interesting to determine the functional consequences of mitochondrial swelling under the different energization statuses, particularly regarding the impact on cytochrome $c$ release as described in our previous publication regarding the use of mitochondria suspended in low ionic strength buffer (33). However, data obtained in KCl buffer consistently showed high basal cytochrome $c$ release in controls that was marginally increased by addition of Cd$^{2+}$ (data not shown; see also Ref. 33). The increase of cytochrome $c$ release in KCl buffer may seem worrisome, because it reflects the physiological situation. First of all, it must be stated that intact mitochondria suspended in KCl buffer do not swell (and hence do not release cytochrome $c$), so that it can be concluded that KCl per se does not damage mitochondria (see Fig. 2). The damaging effect may result from centrifugation of mitochondria at 10,000 g for 5 min in high ionic strength buffer, which is performed after the swelling experiment to separate the mitochondrial pellet from the supernatant. Moreover, salt promotes the release of cytochrome $c$ that is attached to the inner mitochondrial membrane by electrostatic interaction (8). The nature of this cytochrome $c$ pool is not well defined, and it is unclear how it is prevented from being released in vivo.

**What Is the Nature of Cd$^{2+}$-Induced Mitochondrial Contraction?**

Three lines of evidence suggest that a K$^+/H^+$ exchanger is responsible for mitochondrial contraction: 1) contraction does not operate in nonenergized mitochondria (Fig. 1), 2) contraction is abolished by addition of the electroneutral protonophore nigericin at the peak of mitochondrial swelling (Fig. 4), and 3) contraction is abolished by the inhibitor of K$^+/H^+$ exchanger quinine (Fig. 5, A and B). Therefore, the driving force for K$^+/H^+$ exchange must be ΔpH$_{in}$ generated by the respiratory chain. Moreover, pH measurements with BCECF after the addition of 10 μM Cd$^{2+}$ showed dissipation of ΔpH$_{in}$ that was significantly slowed by 1 mM quinine (Fig. 6C). Cd$^{2+}$ indirectly contributes to dissipation of ΔΨ$_{m}$ by inducing K$^+$ influx through a K$^+$ uniporter, which in turn initiates K$^+/H^+$ exchange through the quinine-sensitive K$^+/H^+$ antiporter, with the action of both transporters resulting in K$^+$ cycle and mitochondrial contraction.

**What Are the Consequences of Changes in Membrane Permeability Induced by Cd$^{2+}$ on Mitochondrial Function?**

Other studies have shown that not all changes in mitochondrial function triggered by inducers cause irreversible mito-

Fig. 6. Model showing the Cd$^{2+}$ effects on permeability pathways of the inner mitochondrial membrane. (→, direction of flux; ↑, inhibition; arrowhead, activation). The large voltage-dependent anion channel (VDAC, or porin) is present in the mitochondrial outer membrane that is supposed to be part of the permeability transition pore (PTP) (9, 22). For further details, see text. CsA, cyclosporin A; RR, ruthenium red; AIF, apoptosis-inducing factor; K$_{cc}$ channel, Ca$^{2+}$-stimulated K$^+$-selective channel.
Mitochondrial damage. For instance, Schönfeld et al. (42) showed a decrease of $\Delta \Psi_m$ upon induction of K$^+$ influx by long-chain fatty acids but a recovery of $\Delta \Psi_m$ due to K$^+$ efflux and mitochondrial contraction. Also, temporary changes in mitochondrial volume induced by moderate hypotonicity may lead to cytochrome c release while mitochondria remain intact and functionally active (19). In contrast, the results of this study show that the changes in mitochondrial function induced by Cd$^{2+}$ are detrimental. Cd$^{2+}$ at concentrations $\geq 5 \mu M$ induced a permanent breakdown of $\Delta \Psi_m$ (Fig. 3A). This differs from the findings in the study by Schönfeld et al. (42), in which a decrease of $\Delta \Psi_m$ upon induction of K$^+$ influx by long-chain fatty acids was followed by a recovery of $\Delta \Psi_m$ during the contraction phase. Indeed, Cd$^{2+}$ concentrations $\geq 3 \mu M$ induced a block of O$_2$ consumption within 1–2 min. States 4 and 3 respiration were irreversibly inhibited 2–3 min after addition of Cd$^{2+}$ concentrations $\geq 3 \mu M$ (data not shown), which is in accordance with a recent study demonstrating that 5–20 $\mu M$ Cd$^{2+}$ inhibited mitochondrial complexes II and III of the electron transfer chain (54).

We therefore propose the following working hypothesis to explain the Cd$^{2+}$-induced osmotic swelling-contraction response, changes in K$^+$ permeability pathways, and mitochondrial dysfunction in energized mitochondria (Fig. 6). Cd$^{2+}$ enters the matrix space through the MCU. Both the chemical gradient for K$^+$ as well as $\Delta \Psi_m$ drive K$^+$ influx through a Cd$^{2+}$-activated K$^+$ uniporter. The osmotic load of the mitochondrial space by K$^+$ is directly reflected by the changes in mitochondrial volume over time. The breakdown of $\Delta \Psi_m$ induced by K$^+$ influx and the inhibition of the respiratory chain by Cd$^{2+}$ finally limit K$^+$ influx and lead to a K$^+$ equilibrium across the IMM. K$^+$ in the matrix space triggers activation of a K$^+/H^+$ exchanger in the IMM that dissipates $\Delta$H$_m$ and decreases the K$^+$ concentration and hence the osmotic load of the matrix space, resulting in mitochondrial contraction. Thus the K$^+$ load of the matrix space determines the rate and magnitude of dissipation of $\Delta$H$_m$ via the K$^+/H^+$ exchanger. Consequently, apart from Cd$^{2+}$-induced inhibition of the electron transfer chain (54), Cd$^{2+}$-induced activation of a K$^+$ cycle also affects mitochondrial damage by contributing to the dissipation of the mitochondrial protonotive force.

Independently of the complexity of these alterations of mitochondrial permeability and function induced by Cd$^{2+}$, a major event associated with Cd$^{2+}$-induced mitochondrial swelling remains the release of proapoptotic factors such as cytochrome c (33). Once released into the cytosol, cytochrome c typically forms an apoptosome with other cytosolic components and triggers activation of a cellular cascade of proteases that ultimately leads to apoptosis. It is likely that these processes are operative in Cd$^{2+}$-induced apoptosis of PT cells (49–51), and these processes are currently under investigation.

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