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Characterization by Hg^{2+} of two different pathways for mitochondrial Ca^{2+} release

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The addition of Hg^{2+} to loaded kidney mitochondria induces the fast release of the accumulated cation. The Ca^{2+}-efflux reaction exhibits kinetics characteristics that depend on the extent of the binding of Hg^{2+} to the membrane. At high levels of Hg^{2+} bound (approx. 11 nmol/mg), Ca^{2+} efflux rate is highly insensitive to the temperature of incubation, and the efflux seems to be directly related to the internal free Ca^{2+} concentration. At these levels of bound Hg^{2+}, accumulated Sr^{2+} is released with characteristics similar to those observed with Ca^{2+}. At lower levels of Hg^{2+} binding (2.5 nmol/mg), the efflux reaction is highly dependent on the incubation temperature and on the internal free Ca^{+} concentration; under these conditions Sr^{2+} is not released. NAD(P)H oxidation as induced by the low Hg^{2+} concentration is inhibited at the lower temperatures. Radiolabeled Hg^{2+} incorporates into two clearly defined regions of membrane proteins separated through sodium dodecyl sulfate gel electrophoresis. One of the regions corresponds to proteins of apparent high molecular mass (i.e., 150 kDa), and the other to proteins with apparent molecular masses of 37–25 kDa. Mitochondria incubated with 2 μM $^{203}Hg^{2+}$ incorporate the radionuclide in proteins that have molecular masses of around 41 and 26 kDa. The results indicate that, depending on the amount of Hg^{2+} bound to the inner membrane, two clearly distinct Ca^{2+} release mechanisms can be distinguished.

Introduction

The mechanism by which Ca^{2+} is released from mitochondrial matrix is a widely studied and controversial subject. It is insensitive to Ruthenium red and, as suggested by Fiskum and Cockrell [1], the release may involve a Ca^{2+}/H^{+} antiport, or, as proposed by Crompton et al. [2], a Ca^{2+}–Na^{+} exchange reaction. In addition to the exchange reactions, Ca^{2+} efflux and release of other low-molecular-weight solutes may occur through a mechanism that can be regulated by the redox state of pyridine nucleotides [3–8] and membrane -SH groups [9–11]. In this respect, it has been reported that organic mercurials and other thiol-binding agents can induce Ca^{2+} release from mitochondria, i.e., Palmer and Pfeiffer [12], Broekemeier et al. [13], and Riley and Pfeiffer [14] proposed that *N*-ethylmaleimide- and diamide-induced Ca^{2+} release was due to a phospholipase A_2-dependent permeabilization of the inner membrane. Also, Chávez et al. [15] reported that Hg^{2+} at concentrations higher than 5 μM induces Ca^{2+} efflux, but at low Hg^{2+} concentrations (2 μM), Ca^{2+} efflux exhibits a requirement for dithiothreitol.

The present study was undertaken to ascertain whether Ca^{2+} efflux as induces by high and low Hg^{2+} concentrations operates through different mechanisms. By studying the characteristics of Ca^{2+} efflux as induced by two concentrations of Hg^{2+}, at different temperatures, and the release of Ca^{2+} and Sr^{2+} under the influence of Hg^{2+}, it became apparent that there are two clearly different mechanisms for Ca^{2+} release. Moreover, it was observed that in the presence of oxalate the rate of Ca^{2+} efflux depends markedly on the concentration of Hg^{2+} employed, and that the different characteristics of Ca^{2+} release at high and low Hg^{2+} concentrations are associated with the binding of Hg^{2+} to different membrane proteins.

Materials and Methods

Mitochondria from rat kidney were isolated as described [16]. Protein was determined by the method of Lowry et al. [17]. Changes in Ca^{2+} or Sr^{2+} concentra-

Abbreviations: DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

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tion in the suspending medium were monitored with purified Arsenazo III [18] in a dual-wavelength spectrophotometer at 685–675 nm. Alternatively, Ca^{2+} uptake and release were assayed by the filtration technique, using $^{45}CaCl_2$ (spec. act. 1000 cpm/nmol). Intramitochondrial free Ca^{2+} concentration was determined by using the ionophore A23187, as reported in Ref. 19. The binding of Hg^{2+} to mitochondria was also assayed by the filtration technique by using $^{203}Hg(CH_3COO)_2$ (spec. act. 20000 cpm/nmol). The oxidation–reduction state of mitochondrial pyridine nucleotides was monitored by dual-wavelength spectrophotometry at 370–340 nm. Polyacrylamide gel electrophoresis of mitochondrial proteins labeled with $^{203}Hg^{2+}$ (spec. act. 30000 cpm/nmol), was performed as reported [15]. The distribution of the radioactivity was determined in 2-mm slices of the gels. The composition of the suspending media and other experimental details are given in the individual experiments.

Results

The effect of temperature on Hg^{2+}-induced Ca^{2+} release

The effect of 10 μM and 2 μM plus dithiothreitol (DTT) on mitochondrial Ca^{2+} efflux, at temperatures of 25°C and 15°C was tested (Fig. 1). In agreement with previous data [15], it was observed that at a temperature of 25°C, a rapid Ca^{2+} release occurred upon the addition of 10 μM Hg^{2+} or after the addition of 2 μM Hg^{2+} plus DTT (Fig. 1A). When the experiment was

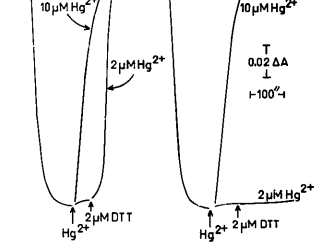

Fig. 1. Effect of temperature on mitochondrial Ca^{2+} release induced by Hg^{2+}. Mitochondria (M, 2 mg protein) were incubated in 3 ml of media containing 250 mM sucrose/10 mM succinate/10 mM Hepes/10 mM acetate/5 μM ADP; 50 μM $CaCl_2$/10 μg rotenone/5μg oligomycin/50 μM Arsenazo III. The media were adjusted to pH 7.3 with KOH. Incubation temperature as indicated. Other additions were made where indicated in the Fig.

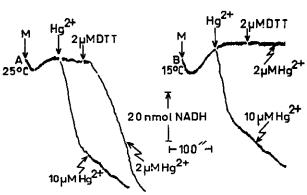

Fig. 2. The influence of temperature on the pyridine nucleotides oxidation induced by Hg^{2+}. Mitochondria, (M, 2 mg protein) were suspended in the standard media described in the legend of Fig. 1 (no Arsenazo III was added). Further additions and incubation temperature were as indicated.

carried out at a temperature of 15°C (Fig. 1B), 10 μM Hg^{2+} produced a fast efflux of accumulated Ca^{2+}, but 2 μM Hg^{2+} plus DTT failed to bring about Ca^{2+} release.

The effect of temperature on Hg^{2+}-induced NAD(P)H oxidation

It has been postulated that the NAD(P)H/NAD(P) ratio is the main regulatory factor of mitochondrial Ca^{2+} release or retention [3–8,20]. Therefore, we examined whether the temperature-dependent Hg^{2+}-induced Ca^{2+} efflux was accompanied by changes of intramitochondrial pyridine nucleotides (Fig. 2). When mitochondria were incubated at 25°C (Fig. 2A), 10 μM Hg^{2+} induced a rapid oxidation of NAD(P)H (17 nmol/mg in the first min). The addition of 2 μM Hg^{2+} plus DTT also promoted a fast NAD(P)H oxidation (9 nmol/mg in the first min). However, when the incubation temperature was diminished to 15°C (Fig. 2B), the oxidation of NAD(P)H depended on the concentration of added Hg^{2+}, i.e., the addition of 10 μM Hg^{2+} caused significant pyridine nucleotides oxidation (15 nmol/mg in the first min), whereas no NAD(P)H oxidation was observed after the addition of 2 μM Hg^{2+} plus DTT.

Dependence of Hg^{2+}-induced Ca^{2+} release on the matrix free Ca^{2+} concentration

Further insight into the nature of the Hg^{2+}-induced Ca^{2+} with or without DTT release was obtained by investigating Ca^{2+} efflux at different concentrations of internal exchangeable Ca^{2+}. To this purpose increased concentrations of oxalate were added to the incubation medium in order to achieve, through chelation of internal Ca^{2+}, different amounts of intramitochondrial Ca^{2+}. Fig. 3 shows that Ca^{2+} release as induced by Hg^{2+} with or without (DTT) depends on the concentration of oxalate introduced; at 6 mM oxalate, 10 μM Hg^{2+} promoted the release of 38 nmol Ca^{2+}/mg; whereas with 2 μM Hg^{2+} + 2 μM DTT, 1nmol Ca^{2+}/mg was released. With 10 μM Hg^{2+}, half-maximal inhibition of

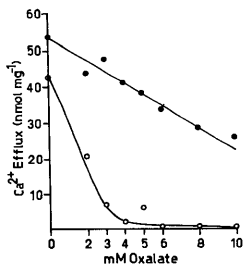

Fig. 3. The effect of increasing concentrations of oxalate on mitochondrial Ca^{2+} release induced by Hg^{2+}. 2 mg protein from mitochondria were incubated in similar conditions to those described for Fig. 1, except that $^{45}Ca^{2+}$ was used, and the indicated concentrations of oxalate. After 2 min of incubation time, aliquots of 0.2 ml were filtered for estimation of accumulated cation. Immediately, 10 μM Hg^{2+} (●), or 2 μM Hg^{2+} plus 2 μM DTT (○) were added. After 2 min, aliquots of 0.2 ml were filtered for estimation of $^{45}Ca^{2+}$ release. Temperature 25 °C.

Ca^{2+} release was obtained with around 5 mM oxalate, whereas, with 2 μM Hg^{2+} plus DTT, the $K_{0.5}$ of the inhibition of Ca^{2+} release was attained with 2 mM oxalate. The findings suggest that the Ca^{2+}-release pathway activated by the DTT-dependent Hg^{2+} binding requires a higher internal free Ca^{2+} concentration than the pathway that becomes apparent with 10 μM Hg^{2+}.

In order to estimate the amount of internal exchangeable Ca^{2+}, the experiment shown in Fig. 4 was carried out. After incubation of mitochondria at different oxalate concentrations, 0.5 μM Ruthenium red and 2 μM of the ionophore A23187 were added. It was assumed that the amount of Ca^{2+} liberated by the ionophore is directly related to the concentration of exchangeable Ca^{2+} [19]. As expected, Ca^{2+} release followed an inverse linear relation with increasing oxalate concentrations. Without oxalate added, 52 nmol Ca^{2+}/mg were released, whereas in the presence of 10 mM oxalate, the release attained was 29 nmol/mg. On the basis of the observations in Figs. 3 and 4, it was possible to establish a molar relationship between the amount of exchangeable mitochondrial Ca^{2+} and the amount of Ca^{2+} released by 10 μM Hg^{2+} or 2 μM Hg^{2+} plus DTT. Release of Ca^{2+} promoted by 10 μM Hg^{2+} followed a linear dependence with the intramitochondrial exchangeable Ca^{2+} (Fig. 5). It was also found that Ca^{2+} efflux, as induced by 2 μM Hg^{2+} plus DTT, became apparent only when the concentration of internal Ca^{2+} was higher than 40 nmol/mg. A notable feature of the release induced by 2 μM Hg^{2+} plus DTT is the cooperative pattern followed by Ca^{2+} release (value of n close to 10). Therefore it appears that, under such conditions, the opening of a passage for Ca^{2+} loss involves a conformational transition which can be induced by the cooperative binding of internal Ca^{2+}. In addition, considering an activity coefficient value of $7 \cdot 10^{-4}$ for intramitochondrial Ca^{2+} [19], the K_m for Ca^{2+} obtained for this efflux reaction would be about 35 μM. This value is similar to that previously calculated for the efflux of Ca^{2+} from heart mitochondria, i.e., 10–15 μM [21].

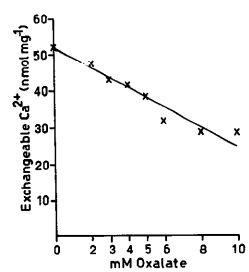

Fig. 4. The relationship between increasing concentrations of oxalate and Ca^{2+} release induced by the ionophore A23187. Experimental procedures were similar to those described in the legend to Fig. 3, except that after 2 min of incubation time 0.5 μM Ruthenium red and 2 μM A23187 were added instead of Hg^{2+} to induce Ca^{2+} release.

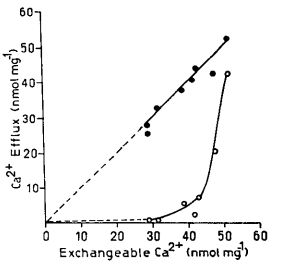

Fig. 5. The relationship between mitochondrial free-Ca^{2+} concentration and Ca^{2+} release induced by Hg^{2+}. The points shown are from data obtained from experiments described in Figs. 3 and 4. ○, 2 μM Hg^{2+} plus 2 μM DTT; ●, 10 μM Hg^{2+}.

The reported affinity-constant value of oxalate for Hg^{2+} may approach 10^4 [22], whereas the estimated affinity constant of Hg^{2+} for mitochondrial sulfhydryl groups is $1.5 \cdot 10^8$ [15]. However, given the high con-

TABLE I

Mercury binding to mitochondrial membrane in the presence of oxalate

The experimental conditions were essentially as described for Fig. 3, except that radiolabeled mercury was used.

Oxalate added (mM)	Hg^{2+} bound (nmol per mg)	
	10 μM Hg^{2+}	2 μM Hg^{2+} + 2 μM DTT
–	11.2	2.4
5	11.1	2.5
10	11	2.4

centration of oxalate added, the chelating effect of Hg^{2+} by oxalate must be evaluated. Accordingly, the experiment shown in Table I was performed; the binding of Hg^{2+} to the membrane reached similar values in the absence or in the presence of 10 mM oxalate, i.e., 11 and 2.4 nmol/mg with 10 and 2 μM Hg^{2+} plus DTT, respectively. This indicates that under our conditions a chelating effect of oxalate, that modifies the binding of Hg^{2+} to the mitochondrial membrane, may be excluded.

The effect of Hg^{2+} on membrane permeability to Sr^{2+}

The higher concentration of internal Ca^{2+} required for operating Ca^{2+} release upon addition of 2 μM Hg^{2+} plus 2 μM DTT (see Fig. 5), suggested the existence of an internally low affinity efflux system for Ca^{2+}. Thus, considering the differences in the ionic radius for Ca^{2+} (0.99 Å), and Sr^{2+} (1.13 Å), the characteristics of Sr^{2+} release from mitochondria were studied to examine a possible ionic selectivity of the two postulated Ca^{2+} releasing pathways. Accumulated Sr^{2+} was rapidly and almost completely released after addition of 10 μM Hg^{2+} (Fig. 6A). In contrast, 2 μM Hg^{2+} plus DTT did not bring about release of Sr^{2+} (Fig. 6B). These data indicate that the pathway for Ca^{2+} efflux which is open at low Hg^{2+} concentrations (plus DTT), but not that which is open at 10 μM Hg^{2+}, has a high specificity for Ca^{2+}. The possibility of a competition between Sr^{2+} and Hg^{2+} for membrane binding sites was also considered. However, the results of Table II indicate that the extent of binding of Hg^{2+} (with or without DTT) was nearly the same in media that contained Ca^{2+} or Sr^{2+}.

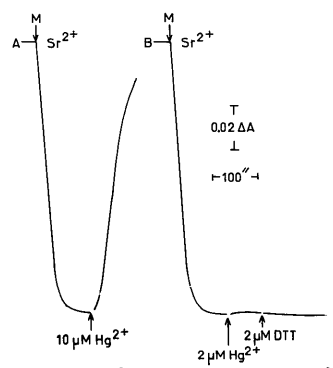

Fig. 6. The effect of Hg^{2+} on the mitochondrial accumulated Sr^{2+}. Experimental conditions were similar to those described for Fig. 1, except that 50 μM $SrCl_2$ was used instead $CaCl_2$. Other conditions were made as indicated. Temperature, 25 °C.

TABLE II

Mercury binding to mitochondrial membrane in the presence of Ca^{2+} and Sr^{2+}

Experimental conditions similar to those described for Figs. 1 and 6, except that 2 μM radiolabeled Hg^{2+} was used.

Additions	Hg^{2+} bound (nmol/mg)
Ca^{2+} + Hg^{2+}	2.1
Ca^{2+} + Hg^{2+} + DTT	2.5
Sr^{2+} + Hg^{2+}	2.0
Sr^{2+} + Hg^{2+} + DTT	2.6

Electrophoretic analysis of ^{203}Hg-labeled membrane proteins

To obtain additional insight into the phenomenon of Hg^{2+}-induced mitochondrial Ca^{2+} release, an electrophoretic analysis of membrane proteins labeled with $^{203}Hg^{2+}$ was performed. The radioactive profile of the proteins of mitochondria previously exposed to 10 μM

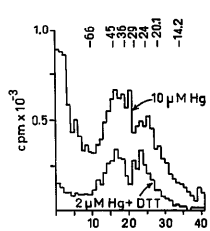

Fig. 7. Electrophoretic analysis of membrane proteins labeled with $^{203}Hg^{2+}$. 2 mg of mitochondrial protein were incubated under similar conditions to those described for Fig. 1. After 5 min incubation the media were layered in 35 ml of 330 mM sucrose and spun down 10 min at 34000 × g. The pellets were treated with 2% SDS without 2-mercaptoethanol, and 200 μg of protein were electrophoresed.

$^{203}Hg^{2+}$ or 2 μM $^{203}Hg^{2+}$ plus DTT as analyzed by SDS gel electrophoresis is shown in Fig. 7. When mitochondria were incubated with 10 μM Hg^{2+}, three peaks of radioactivity became apparent in the gels; one lay in the region of more than 150 kDa, and two other in regions of 37 and 25 kDa. In contrast, mitochondria labeled with 2 μM $^{203}Hg^{2+}$ + DTT exhibit mainly two radioactive peaks, one of approx. 41 kDa and other of 26 kDa.

Discussion

On the basis of several observations, it has been concluded that sulfhydryl-blocking reagents produce an unspecific permeability that results in Ca^{2+} efflux [12–14,23]. The present study establishes that two distinct pathways for Ca^{2+} release can be distinguished by control of the amount of Hg^{2+} that binds to the inner membrane. One pathway is activated when 11 nmol Hg^{2+}/mg bind to the membrane and results in the efflux of Ca^{2+} and Sr^{2+}. Another pathway, that is activated by the binding of 2.5 nmol Hg^{2+}/mg, allows the efflux of Ca^{2+}, but not of Sr^{2+}; it is highly dependent on the incubation temperature and on the internal concentration of exchangeable Ca^{2+}. This pathway appears to be opened by the binding of Hg^{2+} to a limited number of specific membrane thiols. These findings strongly suggest that there are at least two mechanisms that operate in Ca^{2+} release, a suggestion that is further substantiated by the distinct temperature dependence of Ca^{2+} release at high and low (plus DTT) Hg^{2+} concentrations. The rate of Ca^{2+} release initiated after addition of 10 μM Hg^{2+}, regardless of the temperature of incubation (Fig. 1A and B), suggests a generalized permeability. In this context, early experiments [24,25] have shown that high levels of mercurials bound to the membrane (about 15–20 nmol/mg) produce a massive rearrangement of its components that brings about a passive permeability to a number of cations. In contrast, the strict temperature dependence, observed when the efflux pathway becomes operational with 2 μM Hg^{2+} plus DTT, does not seem to follow the pattern of simple diffusion.

Hunter and Haworth [26] reported that the development of a pathway for Ca^{2+} release correlates to the concentration of internal Ca^{2+}. These authors propose that an increase in matrix Ca^{2+} induces a 'membrane transition' that leads to nonspecific changes in the permeability to Ca^{2+}. Also, Nicholls et al. [21,27] pointed out that high levels of accumulated Ca^{2+} produce gross structural alterations conducive to Ca^{2+} loss. In agreement with those findings, our studies with oxalate establish that Ca^{2+} efflux induced by 10 μM Hg^{2+} follows a direct relation with the level of endogenous Ca^{2+} concentration (Fig. 5). In contrast, when 2 μM Hg^{2+} plus DTT are used, it appears that the chelation of internal Ca^{2+} by oxalate maintains the exchangeable matrix Ca^{2+} below the concentration required to saturate the corresponding efflux pathways. The sigmoidicity of the reaction suggests that the mitochondrial membrane contains units which, after titration by Ca^{2+}, are able to open a transmembrane hydrophilic channel. In this respect, it has been proposed that mitochondria contain hydrophilic channels which become operative upon Ca^{2+} binding [28].

Palmer and Pfeiffer [12] and Pfeiffer et al. [29] indicated that the lack of thiol reagents to induce Sr^{2+} release is due to a failure of this cation to activate phospholipase A_2. On this assumption, it would appear that Sr^{2+} release by 10 μM Hg^{2+} does not involve phospholipase A_2 action. Nevertheless, regardless of the mechanism involved in the opening of this pathway, the important fact is that the efflux pathway induced by the binding of high Hg^{2+} (11 nmol/mg) allows the efflux of Ca^{2+} and Sr^{2+}, whereas the pathway opened by 2 μM Hg^{2+} plus DTT does not result in Sr^{2+} release.

The electrophoretic analysis of ^{203}Hg-labeled proteins of mitochondria is also consistent with the existence of two distinct separate pathways for Ca^{2+} release. The wide distribution of the labeled proteins when mitochondria were incubated with 10 μM Hg^{2+} would correspond to a state of the mitochondria in which there is a generalized permeability. However, when mercury binding is limited to membrane proteins of M_r between 26 and 45 kDa, Ca^{2+} release takes preference over Sr^{2+} release. The radioactivity profile obtained with 2 μM Hg^{2+} plus 2 μM DTT is similar to that reported previously [15], and may involve proteins such as the energy-linked transhydrogenase [30], glutathione reductase [31], phospholipase A_2, and the adenine nucleotide translocase [33]. These membrane proteins have been suggested to regulate intramitochondrial Ca^{2+} levels.

In conclusion, based on the results presented here, we would like to propose that in kidney mitochondria, there are two distinct pathways for Ca^{2+} release, and that these pathways are regulated by the oxidation state of two sets of membrane thiols.

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