The Action of Nupercaine on Calcium Efflux from Rat Liver Mitochondria

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1. Nupercaine inhibits the Ca^{2+} efflux from rat liver mitochondria observed in the presence of Ruthenium Red, 50% inhibition being obtained at $80\mu\text{M}$ -Nupercaine. 2. Neither the Ruthenium Red-stimulated efflux nor its inhibition by Nupercaine can be directly attributed to effects on mitochondrial stability. 3. Nupercaine perturbs the steady-state external Ca^{2+} concentration in the absence of Ruthenium Red to an extent that is explicable in terms of the inhibition of Ca^{2+} efflux. 4. Various factors that are likely to be involved in determining steady-state extra-mitochondrial Ca^{2+} concentrations are discussed.

There have been several reports (e.g. Crompton *et al.*, 1976) suggesting that mitochondria, in addition to the well-documented mechanism for Ca^{2+} uptake, have a separate route for Ca^{2+} efflux. The Na^{+} -stimulated Ca^{2+} efflux found in many types of mammalian mitochondria is absent from liver mitochondria (Crompton *et al.*, 1978). However, many workers have found that when Ruthenium Red, a potent inhibitor of Ca^{2+} uptake (Moore, 1971; Reed & Bygrave, 1974), is added to rat liver mitochondria which are in a steady state with respect to external Ca^{2+} concentration, there is an efflux of Ca^{2+} from the mitochondria (Stucki & Ineichen, 1974; Puskin *et al.*, 1976; Pozzan *et al.*, 1977; Crompton *et al.*, 1978). This finding lends support to the idea that there is an efflux mechanism with a different thermodynamic driving force from that for uptake. Ca^{2+} -phosphate symport (Lötscher *et al.*, 1979) and Ca^{2+} - H^{+} antiport (Fiskum & Lehninger, 1979) have been suggested as candidates for the efflux mechanism.

Nicholls (1978b) has produced convincing evidence that the distribution of Ca^{2+} between intra- and extra-mitochondrial space for liver mitochondria is a steady state, rather than a thermodynamic equilibrium. The extra-mitochondrial Ca^{2+} concentration was found to be essentially constant over a wide range of Ca^{2+} loads and membrane potentials. Since Ruthenium Red was not used in these investigations, the possibility of artefacts being introduced by the presence of the inhibitor was avoided. However, it remains to be established as to whether or not the Ca^{2+} efflux observed after Ruthenium Red addition is the same as the efflux

pathway, which is implied by the results of Nicholls (1978b). We have shown that some local anaesthetics, in particular Nupercaine and Tetracaine, inhibit the Ca^{2+} efflux observed after Ruthenium Red addition in the steady state (Dawson *et al.*, 1979), and are therefore potentially useful in the investigation of the efflux pathway. Local anaesthetics, apart from their stimulatory action on mitochondrial Ca^{2+} uptake (Mela, 1968, 1969), are known to enhance mitochondrial stability (Scarpa & Lindsay, 1972; Aleksandrowicz *et al.*, 1973), which is very probably due to inhibition of endogenous phospholipase A (Seppala *et al.*, 1971; Scherphof *et al.*, 1972). If a fraction of the mitochondrial population were losing their integrity, and hence their matrix Ca^{2+} , during the time course of the experiment, this would be observed either as an effect on the extra-mitochondrial steady-state Ca^{2+} concentration, as the released Ca^{2+} redistributed into the remaining intact mitochondria, or as a net efflux if re-uptake was blocked by Ruthenium Red. It is thus important to assess the stability of the mitochondria under the experimental conditions used to study the efflux pathway, and to examine the effects of local anaesthetics on this stability.

We report here some findings which suggest that the inhibition of Ca^{2+} efflux by Nupercaine is not directly related to effects on the stability of the mitochondria. We also show that, as well as inhibiting Ruthenium Red-promoted efflux, Nupercaine causes a decrease in the steady-state external Ca^{2+} concentration maintained by mitochondria. This indicates that the efflux observed after Ruthenium Red addition is unlikely to be an artefact

produced by the inhibitor and is related to the efflux under steady-state conditions observed by Nicholls (1978b).

Experimental

Materials

All radioactive isotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Sucrose solutions were passed through a column of Dowex chelating resin (Sigma Chemical Co.) to remove endogenous Ca^{2+} before addition of buffers. Ruthenium Red was from BDH, Poole, Dorset, U.K. The concentration of Ruthenium Red solutions was determined by measurement of the A_{532} , by using an A_{mm} of 61.5 (Fletcher *et al.*, 1961). Arsenazo III (grade I) was from Sigma Chemical Co., as were rotenone and HEDTA (*N*-hydroxy-ethylethylenediaminetriacetic acid). Nupercaine (cinchocaine hydrochloride) was a gift from CIBA Laboratories, Horsham, Sussex, U.K. Bovine serum albumin was from Armour Pharmaceuticals, Eastbourne, U.K., and was dialysed against water before use. All other reagents were of analytical or highest available commercial grade.

Methods

Rat liver mitochondria. These were made and their protein concentrations determined as previously described (Selwyn *et al.*, 1970), except that 0.5 mM-EGTA was included in the homogenization and first wash media. Mitochondria were then washed twice in a medium containing 250 mM-sucrose (treated with chelating resin) buffered with 5 mM-Hepes [4-(2-hydroxyethyl-1-piperazine-ethanesulphonic acid)]/KOH, pH 7.6. Endogenous Ca^{2+} content of mitochondria was measured with a Pye-Unicam SP.191 atomic-absorption spectrophotometer after digestion of the samples with H_2SO_4 and HClO_4 . For 11 mitochondrial preparations the endogenous Ca^{2+} content was 14.7 ± 3.6 (s.d.) nmol $\cdot$ mg $^{-1}$.

Ca^{2+} uptake and efflux. Ca^{2+} movements were routinely followed by using Arsenazo III as an indicator for extra-mitochondrial Ca^{2+} concentration (Scarpa, 1975). The instrument used was an Aminco-Chance dual-wavelength spectrophotometer equipped with a stirred circular cuvette of 1 cm diameter. The overall mixing and response time was approx. 0.5 s. The absorbance of Arsenazo III was followed at the wavelength pair 685–665 nm. The experimental medium contained, in a volume of 5 ml: 250 mM-sucrose, 10 mM-Tris/HCl, pH 7.6, 2 μ g of rotenone, 8 μ M-Arsenazo III and 0.5–2.0 mg of mitochondrial protein $\cdot$ ml $^{-1}$. Subsequent additions were made with the aid of a syringe through a small hole in the lid of the cell housing. The temperature was 30°C.

Initial rates of Ca^{2+} uptake were measured in a medium similar to the above with the addition of 0.1 mM-HEDTA (Tris salt). CaCl_2 was added to give a free Ca^{2+} concentration of 3.12 μ M, measured with a Ca^{2+} -sensitive electrode (Radiometer, Copenhagen, Denmark), and uptake was started by the addition of 2.5 mM-succinate (K^+ salt).

Alternatively, Ca^{2+} uptake and efflux were followed radioisotopically, by using $^{45}\text{Ca}^{2+}$. The experimental medium used contained, in a volume of 5 ml: 250 mM-sucrose, 10 mM-Tris/HCl, pH 7.6, 2 μ g of rotenone, 0.5–2.0 mg of mitochondrial protein $\cdot$ ml $^{-1}$ and 5 μ M added CaCl_2 , containing 0.4 μ Ci of $^{45}\text{Ca}^{2+}$ $\cdot$ ml $^{-1}$. After equilibration for 4 min at 30°C, uptake was initiated by the addition of 2.5 mM-potassium succinate. Samples (0.3 ml) of the incubation mixture were centrifuged for 1 min at 15 000 *g* in an Eppendorf 3200 centrifuge, followed by removal of 0.1 ml samples of the supernatant for scintillation counting in 15 ml of scintillant. The scintillant used contained, in 1 litre, 4.67 g of 2,5-diphenyloxazole, 660 ml of toluene and 340 ml of Triton X-100. For some experiments, instead of centrifugation, the mitochondria were separated from the medium by rapidly forcing (2 s) 1 ml samples of the incubation mixture through 0.45 μ m Millipore filters mounted on Swinnex holders (Millipore). Samples of the filtrate were then taken for scintillation counting.

Following the observations of Dargel (1974), mitochondria were always added to a medium already containing rotenone.

Measurement of extra-mitochondrial steady-state Ca^{2+} concentration. Since Nupercaine was found to produce severe interference with the Ca^{2+} -sensitive electrode, it was necessary to adapt the Arsenazo III technique for measurement of this parameter. The incubation mixture contained, in a volume of 5 ml: 0.1 M-NaCl, 10 mM-Hepes/NaOH, pH 7.0, 16 μ M-bovine serum albumin, 2 μ g of rotenone, 20 μ M-Arsenazo III, 1.0 mg of mitochondrial protein $\cdot$ ml $^{-1}$, and 100 μ M-Nupercaine when required. Uptake of Ca^{2+} was started by the addition of 2.5 mM-succinate. CaCl_2 was then added to give the required loading. When the steady state had been established, indicated by a constant value for the absorbance difference 685–665 nm, 10 μ l of 20 mM-EGTA (Tris salt, pH 7.0) was added. The rapid change in absorbance as residual free Ca^{2+} was chelated by the EGTA was taken as a measure of the free Ca^{2+} concentration. Calibration was by the addition of known concentrations of CaCl_2 solutions (1.0 or 2.0 μ M final concn.) to complete reaction media supplemented with 2 nmol of Ruthenium Red $\cdot$ mg $^{-1}$ to block re-uptake of the added Ca^{2+} .

Measurement of proton electrochemical gradient. This was measured by the method of Nicholls (1974). The experimental medium contained, in a

volume of 5 ml: 250 mM-sucrose, 10 mM-Tris/HCl, pH 7.6, 0.5 mM-KCl, 10 μM - $^{86}\text{RbCl}$ (0.025 $\mu\text{Ci/ml}$), 6 μM - ^{14}C -methylamine (0.25 $\mu\text{Ci/ml}$), 10 μM - ^3H -acetate (3 $\mu\text{Ci/ml}$), 0.5 μg of valinomycin $\cdot\text{ml}^{-1}$, 0.4 μg of rotenone $\cdot\text{ml}^{-1}$ and 2 mg of mitochondrial protein $\cdot\text{ml}^{-1}$. After 2 min preincubation, 2.5 mM-Tris/succinate was added. Then 150 μl samples were filtered rapidly through 0.45 μm Millipore filters. The filters were placed in scintillation vials and counted for radioactivity on a Packard Tri-Carb scintillation counter as described by Nicholls (1974). The intramitochondrial space was assumed to be 0.4 $\mu\text{l} \cdot \text{mg}$ of protein $^{-1}$ (Nicholls, 1974).

Measurement of Ca^{2+} movements under the conditions of membrane-potential measurements was made by two methods. (1) The medium used for determination of membrane potential was supplemented with 8 μM -Arsenazo III and movement of endogenous Ca^{2+} followed in the dual-wavelength spectrophotometer. (2) The radiolabelled Rb^+ , methylamine and acetate in the medium were replaced with an equivalent concentration of the unlabelled compounds and the mixture was supplemented with 0.27 μCi of $^{45}\text{CaCl}_2$ (0.5 $\mu\text{Ci} \cdot \text{nmol}^{-1}$) $\cdot\text{ml}^{-1}$. Extra-mitochondrial Ca^{2+} was determined by scintillation counting of samples of the supernatant after rapid centrifugation (15 000 $g \cdot \text{min}$).

Results

Fig. 1 shows the effect of various Nupercaine concentrations on the initial rate of Ca^{2+} uptake and also on Ca^{2+} release after Ruthenium Red addition. The results are qualitatively very similar to those previously described for Tetracaine (Dawson *et al.*, 1979), with 50% inhibition of efflux being obtained at approx. 80 μM -Nupercaine. The anaesthetic has very little effect on the kinetics of uptake up to a concentration of about 200 μM . Above this concentration uptake is inhibited. Since these high concentrations of Nupercaine also cause swelling and release of respiratory control, the inhibition of uptake can probably be attributed to structural damage to the mitochondrial membrane. It should be noted that the starting Ca^{2+} concentration for the uptake experiments (3 μM) was chosen such that effects either on K_m or V_{max} of the uptake system should have been observed.

The extent of Ca^{2+} efflux after Ruthenium Red addition is considerable. The data shown in Fig. 2, in agreement with those of Crompton *et al.* (1978), show that up to 50% of the accumulated Ca^{2+} is released within 3 min of the addition of Ruthenium Red. Fig. 2 also shows that Nupercaine produces a very similar degree of inhibition when efflux is measured by using $^{45}\text{Ca}^{2+}$ to that measured by using the Arsenazo III method. The large scale of the

Fig. 1. Effect of Nupercaine concentration on velocity of Ca^{2+} uptake and efflux

Changes in extra-mitochondrial Ca^{2+} concentrations were measured by using Arsenazo III as described under 'Methods'. The assay system for efflux (●) contained in a volume of 5 ml: 250 mM-sucrose, 10 mM-Tris/HCl, pH 7.6, 2 μg of rotenone, 8 μM -Arsenazo III, 0.74 mg of mitochondrial protein $\cdot\text{ml}^{-1}$ and 21 μM total Ca^{2+} . Uptake was started by the addition of 2.5 mM-succinate and, once uptake was complete, efflux was observed after the addition of 1.5 nmol of Ruthenium Red $\cdot\text{mg}$ of protein $^{-1}$. The kinetics of uptake (○) were followed under the same conditions, except that 0.1 mM-HEDTA (Tris salt) was present, and the starting free Ca^{2+} concentration was 3.12 μM .

efflux process observed on Ruthenium Red addition makes it unlikely that mitochondrial fragility is directly involved. The data of Fig. 2 would require that approx. 50% of the mitochondria break up every 3 min. However, because of the well-documented effects of Nupercaine on mitochondrial stability, it is important to establish that the mitochondria remain energetically competent over the sort of time scale used in the experiment shown in Fig. 2 and under similar conditions. Fig. 3 shows measurement of membrane potential and pH gradient in the presence and absence of Ruthenium Red and 80 μM -Nupercaine. Both the total protonmotive force and the individual contributions of membrane potential and pH gradient are constant after the first 90 s from the addition of substrate and are essentially unaffected by the addition of Nupercaine or Ruthenium Red. Since this experiment should measure the energy state of the whole population, it seems to rule out the possibility that the inhibition of efflux produced by Nupercaine in the presence of Ru-

Fig. 2. Effect of Nupercaine on efflux of $^{45}\text{Ca}^{2+}$ from mitochondria

Measurements were made as described under 'Methods', by using rapid centrifugation to separate mitochondria from the medium. The mitochondrial protein concentration was $1.4 \text{ mg} \cdot \text{ml}^{-1}$. At $t = 0$, 2.5 mM -succinate was added: O, no further addition; ●, 1.5 nmol of Ruthenium Red $\cdot \text{mg}$ of protein $^{-1}$ added at (b); (Δ) $100 \mu\text{M}$ -Nupercaine added at (a), followed by 1.5 nmol of Ruthenium Red $\cdot \text{mg}^{-1}$ at (b).

Fig. 4. Ca^{2+} movements under the conditions used for membrane-potential measurements

Conditions were the same as those used for the experiment of Fig. 3, except that radioactive Rb^{+} , methylamine and acetate were replaced with unlabelled compounds, and $0.27 \mu\text{Ci}$ of $^{45}\text{CaCl}_2 \cdot \text{ml}^{-1}$ was added. $^{45}\text{Ca}^{2+}$ distribution was followed by using the rapid-centrifugation technique. Δ, No further additions; O, 1.2 nmol of Ruthenium Red $\cdot \text{mg}$ of protein $^{-1}$ added at the arrow; ●, 1.2 nmol of Ruthenium Red $\cdot \text{mg}$ of protein $^{-1}$ and $80 \mu\text{M}$ -Nupercaine added at the arrow.

Fig. 3. Lack of effect of Ruthenium Red and Nupercaine on membrane potential and pH difference across the mitochondrial membrane

Conditions were as described under 'Methods': O, membrane potential, ΔE ; ●, $-59 \Delta\text{pH}$. (a) No additions; (b) 1.2 nmol of Ruthenium Red $\cdot \text{mg}$ of protein $^{-1}$ added at (1); (c) $80 \mu\text{M}$ -Nupercaine added at (2); (d) 1.2 nmol of Ruthenium Red $\cdot \text{mg}$ of protein $^{-1}$ and $80 \mu\text{M}$ -Nupercaine added at (3). Each point represents the average of two determinations on different mitochondrial preparations.

thenium Red is directly related to effects on mitochondrial stability. It is also apparent that the inhibition is not due to alterations in the contributions of membrane potential and pH gradient to the total protonmotive force. However, since the conditions used for membrane-potential measurements are rather different from those used previously to look at the effect of Nupercaine on Ca^{2+} efflux, the experiment shown in Fig. 4 was carried out. This shows that under conditions identical with those used for membrane-potential measurements, but with the addition of tracer quantities of $^{45}\text{Ca}^{2+}$, Ruthenium Red still promotes Ca^{2+} efflux, and this efflux is inhibited by Nupercaine.

The demonstration of the inhibitory action of Nupercaine in these experiments has depended on the prior addition of Ruthenium Red. However, it is clear that if the Ca^{2+} efflux observed after Ruthenium Red addition is not an artefact induced by the inhibitor, the steady-state external Ca^{2+} concentration should be perturbed by adding an inhibitor of the efflux process. The conditions used by Dawson *et al.* (1979) and in the experiments shown in Figs. 1 and 2 are such that the external steady-state Ca^{2+} concentration is decreased to very low values, making accurate measurements very difficult. However, if the external Ca^{2+} concentration is raised by the addition of acetoacetate in the presence of phosphate to enhance efflux (Lehninger *et al.*, 1978), the addition of Nupercaine causes the re-uptake of Ca^{2+} (Fig. 5). This observation is

consistent with the view that Nupercaine inhibits the efflux process in the absence of Ruthenium Red. To measure the effect of Nupercaine on the steady-state Ca^{2+} concentration in the absence of acetoacetate it was necessary to use the conditions developed by Nicholls (1978*b*). Fig. 6 shows the effect of $100\mu\text{M}$ -Nupercaine on the steady-state external Ca^{2+} concentration in a medium containing 100mM -NaCl and $16\mu\text{M}$ -serum albumin at pH 7.0. In the absence

Fig. 6. Effect of Nupercaine on external steady-state Ca^{2+} concentration at different Ca^{2+} loads. Conditions were as described under 'Methods'. O, Control; ●, plus $100\mu\text{M}$ -Nupercaine.

Fig. 5. Effect of Nupercaine on external steady-state Ca^{2+} concentration

The experimental medium contained, in 5 ml: 250mM -sucrose, 10mM -Tris/HCl, pH 7.6, $1\mu\text{g}$ of rotenone, $8\mu\text{M}$ -Arsenazo III, 0.5mM -potassium phosphate and 0.6mg of mitochondrial protein $\cdot\text{ml}^{-1}$. Arsenazo III absorption was followed at $685-665\text{nm}$, and an upward deflection of the trace indicates decreasing extra-mitochondrial Ca^{2+} concentration. Additions: (1), 2.5mM -potassium succinate; (2), $10\mu\text{M}$ - CaCl_2 ; (3), 2.5mM -lithium acetoacetate; (4), $100\mu\text{M}$ -Nupercaine. The dashed portion of the trace shows Ca^{2+} movements in the absence of Nupercaine. The rapid absorbance change on addition of acetoacetate is an addition artefact.

Fig. 7. Effect of mitochondrial Ca^{2+} load on Ca^{2+} -efflux rate

Conditions were the same as those used for the experiment of Fig. 6. Ca^{2+} movements were followed by using Arsenazo III. After Ca^{2+} additions to give the required loading and attainment of a steady state, efflux was started by the addition of 2 nmol of Ruthenium Red $\cdot \text{mg}^{-1}$. O, Control; ●, medium supplemented with 100 μM -Nupercaine.

of Nupercaine, the variation of external pCa^{2+} with Ca^{2+} load is very similar to that reported by Nicholls (1978b). The presence of 100 μM -Nupercaine causes an increase in pCa^{2+} , which varies from about 0.1 unit at low Ca^{2+} loads up to 0.2 unit at loadings of 70 $\text{nmol} \cdot \text{mg}^{-1}$ and greater. Fig. 7 shows, under the same experimental conditions, the effect of Ca^{2+} loading on efflux rate after Ruthenium Red addition. The increase in external steady-state Ca^{2+} concentration with Ca^{2+} load is approximately coincident with the dramatic rise in efflux rate observed at Ca^{2+} loads of over 60 $\text{nmol} \cdot \text{mg}^{-1}$ (Fig. 7). It also appears that inhibition of efflux by Nupercaine is greater at higher Ca^{2+} loads. The data in Fig. 7 show that 100 μM -Nupercaine causes a 45% inhibition of efflux at a loading of 30 $\text{nmol} \cdot \text{mg}^{-1}$, but an approx. 65% inhibition at a loading of 80 $\text{nmol} \cdot \text{mg}^{-1}$. The shape of the curve observed in Fig. 7 is very similar to that reported by Harris *et al.* (1979) for rat heart mitochondria in the presence of mersalyl.

Discussion

The observations in the present paper are consistent with the view that Ca^{2+} efflux observed after Ruthenium Red addition to Ca^{2+} -loaded mitochondria is a manifestation of an efflux mechanism which is operative all the time. As with mitochondria from

brain and heart (Nicholls, 1978a), efflux in the presence of Ruthenium Red takes place with no diminution in protonmotive force, so that Ca^{2+} loss is not dependent on mitochondrial damage. However, unlike heart and brain mitochondria, efflux from liver mitochondria does not seem to be accompanied by changes in the individual contributions of membrane potential and pH gradient. This observation would suggest that exit of Ca^{2+} is by an electroneutral and pH-balanced process, such as a Ca^{2+} -(anion) $^{2-}_n$ symport. However, other ions may redistribute to maintain a constant membrane potential and ΔpH , so that it is unwise on the basis of this experiment to try to distinguish between Ca^{2+} -anion symport (Lötscher *et al.*, 1979) and Ca^{2+} - H^+ antiport (Fiskum & Lehninger, 1979) as the mechanism for efflux.

The inhibition of efflux by Nupercaine cannot be ascribed directly to changes in mitochondrial fragility caused by phospholipase A activity, since the mitochondria seem to be stable in the absence of the anaesthetic. Similarly, Nupercaine, under the experimental conditions used here, has no effect on the relative contributions of membrane potential and ΔpH to the protonmotive force, so that changes in these parameters are unlikely to be responsible for the inhibition. Local anaesthetics are known to displace Ca^{2+} from mitochondrial membranes (Scarpa & Azzi, 1968). However, there appears to be no evidence of competitive inhibition of Ca^{2+} efflux, as might be expected if Ca^{2+} were being displaced from a carrier by Nupercaine. Indeed, under these conditions, inhibition is somewhat greater at higher Ca^{2+} loadings. The mechanism of inhibition is thus obscure, but may reside in effects on the permeability of the mitochondrial membrane to anions (Selwyn *et al.*, 1978), or in effects on the loss of adenine nucleotides and Mg^{2+} , which appear to be important factors in Ca^{2+} retention (Al-Shaikhly & Baum, 1979; Harris *et al.*, 1979). A non-specific effect on membrane fluidity or thickness is a further possibility.

Whatever the mechanism of inhibition, Nupercaine perturbs the steady-state extra-mitochondrial Ca^{2+} concentration, as would be predicted if a Ca^{2+} cycle were operating (Carafoli, 1979). Nicholls (1978b) found that the rate of Ca^{2+} uptake was decreased by a factor of 2 for each 0.12 unit increase in external pCa^{2+} . Thus it would be expected that a 50% inhibition of efflux would result in an increase of 0.12 in external pCa^{2+} , since at this new value the rate of uptake should also be decreased by 50%. The results shown in Figs. 6 and 7 are consistent with this. A 45% inhibition of efflux (at low loading) causes a shift in pCa^{2+} of 0.1 unit, and a 65% inhibition (at high loading) causes a shift of 0.2 unit. The calculated values are 0.11 and 0.19 respectively.

It appears that a contributory factor to the decreased external pCa^{2+} at high loading could be the greatly increased efflux rate under these conditions (Fig. 7). However, it is only at these high loadings that there is any relationship between the distribution ratio for Ca^{2+} in extra- and intra-mitochondrial space and mitochondrial membrane potential (Nicholls, 1978b). This apparent anomaly would be resolved if it is assumed that the activity coefficient of intra-mitochondrial Ca^{2+} is not constant at different Ca^{2+} loadings. It is known that liver mitochondria contain binding sites for Ca^{2+} (see, e.g., Rossi *et al.*, 1967) and, in addition, endogenous phosphate and bicarbonate ions will lead to intra-mitochondrial precipitation of Ca^{2+} . At low Ca^{2+} loading, precipitation and binding of Ca^{2+} would result in a very small activity coefficient for accumulated Ca^{2+} . Under these conditions, there would be apparently no dependence of accumulation ratio on membrane potential, and low efflux rates. At higher loading, when the binding sites and precipitable anions are saturated, the activity coefficient would rise rapidly, resulting in both dependence of accumulation ratio on membrane potential and enhanced efflux. Thus it would be expected that, at high loading, although the accumulation ratio might be dependent on membrane potential, it would not be in equilibrium with it, since efflux is fast under these conditions.

The value of the activity coefficient for intra-mitochondrial Ca^{2+} is likely to depend on intra-mitochondrial pH (Rossi *et al.*, 1967) and will also be affected by the presence of Nupercaine (Low *et al.*, 1979). The external steady-state pCa^{2+} that will result at a given Ca^{2+} loading is thus dependent on these factors as well as membrane potential and efflux rate. These complexities may afford an explanation of the rather strange phenomenon shown in Fig. 7, where, in the presence of Nupercaine, increasing the Ca^{2+} load from 30 to 50 nmol·mg⁻¹ results in an increased external pCa^{2+} .

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