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The Regulation of Brain Mitochondrial Calcium-Ion Transport

THE ROLE OF ATP IN THE DISCRIMINATION BETWEEN KINETIC AND MEMBRANE-POTENTIAL-DEPENDENT CALCIUM-ION EFFLUX MECHANISMS

David G. NICHOLLS and Ian D. SCOTT
Neurochemistry Laboratory, Department of Psychiatry, Ninewells Medical School, University of Dundee, Dundee DD1 9SY, Scotland, U.K.

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Mitochondria from guinea-pig cerebral cortex incubated in the presence of P_i or acetate are unable to regulate the extramitochondrial free Ca^{2+} at a steady-state which is independent of the Ca^{2+} accumulated in the matrix. This is due to the superimposition on kinetically regulated Ca^{2+} cycling of a membrane-potential-dependent reversal of the Ca^{2+} uniporter. The latter efflux is a consequence of a low membrane potential, which correlates with a loss of adenine nucleotides from the matrix. Low concentrations of ATP prevent adenine nucleotide loss from the matrix, enable the mitochondria to maintain a high membrane potential and allow the mitochondria to buffer the extramitochondrial free Ca^{2+} precisely when up to 200nmol of Ca^{2+}/mg of protein is accumulated in the matrix. The steady-state extramitochondrial free Ca^{2+} is maintained as low as 0.3μM. The Na^+-activated efflux pathway is functional in the presence of ATP and oligomycin and accounts precisely for the change in steady-state free Ca^{2+} induced by Na^+ addition. The need to distinguish carefully between kinetic and membrane-potential-dependent efflux pathways is emphasized and the competence of brain mitochondria to regulate cytosolic free Ca^{2+} concentrations *in vivo* is discussed.

Mitochondria from a variety of tissues have been shown to possess not only a uniport for the uptake of Ca^{2+}, but also a Ruthenium Red-insensitive pathway, which is Na^+-independent in liver and Na^+-activated in heart and brain, and which allows a continuous Ca^{2+} efflux to occur even when $\Delta\psi$ is high (Vasington *et al.*, 1972; Sordahl, 1974; Stucki & Ineichen, 1974; Crompton *et al.*, 1976, 1978; Puskin *et al.*, 1976; Azzone *et al.*, 1977; Nicholls, 1978*a,b*; Åkerman, 1978; Crompton & Heid, 1978; Caroni *et al.*, 1978; Lehninger *et al.*, 1978; Lötscher *et al.*, 1979; Fiskum & Lehninger, 1979). The resultant steady-state cycling of Ca^{2+} across the inner mitochondrial membrane allows the mitochondrion to regulate the extramitochondrial free Ca^{2+} concentration with a precision and flexibility that would not be possible with a single carrier (Nicholls, 1978*b,c*), and enhances the possibility that a general function of mitochondria *in vivo* is in the regulation of cytosolic Ca^{2+} (Bygrave, 1978; Carafoli & Crompton, 1978).

Investigations of Ca^{2+} cycling by mitochondria *in vitro* are complicated by the existence of two independent mechanisms whereby a net efflux of Ca^{2+} can be observed. The first is kinetic, and is operative when $\Delta\psi$ is sufficiently high (i.e. greater than 120mV; Nicholls, 1978*b*) for the uniporter to be essentially irreversible in the direction of Ca^{2+} uptake. Under these conditions Ca^{2+} efflux occurs through the separate efflux pathway, and a net loss of Ca^{2+} from the matrix thus occurs when the uniporter is partially inhibited, or when the efflux pathway is activated for example by the addition of Na^+ to heart or brain mitochondria (Crompton *et al.*, 1976, 1978; Nicholls, 1978*a,b*).

The second efflux mechanism is thermodynamic in nature, and is only observed when $\Delta\psi$ decreases sufficiently for the uniporter to become reversible and allow efflux to occur. Clearly this membrane-potential-dependent mechanism does not depend on a separate efflux pathway, and thus provides no evidence for Ca^{2+} cycling. As discussed previously

Abbreviations used: pCa_0^{2+}, the negative logarithm of the free Ca^{2+} concentration in the extra-mitochondrial (or cytosolic) compartment; Tes, 2-{[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino}ethanesulphonate; $\Delta\psi$, membrane potential; ΔpH, pH gradient; $\Delta\mu_{H^+}$, proton electrochemical potential gradient.

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(Nicholls, 1978*b*), the extent to which $\Delta\psi$ must decrease to observe potential-dependent efflux makes it unlikely that this mechanism is of physiological significance, except possibly during ischaemia. However, if $\Delta\psi$ is not monitored, the distinction between these two mechanisms is not always self-evident. This is particularly so in the presence of physiological concentrations of P_i, when $\Delta\psi$ can decrease spontaneously due to swelling and time-dependent damage to the mitochondria (for reviews see Lehninger *et al.*, 1967; Carafoli & Crompton, 1978; Bygrave, 1978). As low concentrations of ATP or oligomycin (Rossi & Lehninger, 1964) protect mitochondria against this P_i-dependent damage, an efflux that is not observed in the presence of these agents is likely to be a consequence of an artificial collapse in $\Delta\psi$ rather than being due to a truly independent efflux pathway.

In the present paper we observe that brain mitochondria incubated in the presence of physiological concentrations of P_i are unable to buffer pCa_0^{2+} with precision, due to the superimposition of both mechanisms of Ca^{2+} efflux. Artefactual Ca^{2+} efflux due to a decreased $\Delta\psi$ and loss of matrix adenine nucleotides is apparent in the presence of sub-micromolar concentrations of free Ca^{2+}, and is abolished by ATP and oligomycin. However, the Na^+-activated efflux pathway can still be observed in the presence of the nucleotide and oligomycin, and can account precisely for the effect of Na^+ on the steady-state pCa_0^{2+} observed under these conditions.

It is significant first that under these conditions the brain mitochondria can accumulate in excess of 200nmol of Ca^{2+}/mg of protein; secondly that they can precisely regulate the extramitochondrial free Ca^{2+} at concentrations as low as 0.3μM; thirdly that the mitochondria can respond to a transient increase in free Ca^{2+} to 0.6μM with a net uptake of up to 40nmol of Ca^{2+}/min per mg of protein.

As ATP and P_i are usually present in the cytosol in millimolar concentrations (Akerboom *et al.*, 1978) it is suggested that they should be routine constituents of media for the study of physiological aspects of mitochondrial Ca^{2+} transport.

Part of this work has been published as a conference report (Nicholls & Scott, 1979).

Experimental

All radioactive isotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin (fraction V) was obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K., and was exhaustively dialysed against water before use. Ficoll was obtained from Pharmacia, Uppsala, Sweden, and was also dialysed against water. All other reagents were of analytical grade.

Mitochondria

'Free' mitochondria (i.e. those not contained within synaptosomes) were obtained from the cerebral cortices of Duncan–Hartley strain guinea pigs of either sex as previously described (Nicholls, 1978*a*). The mitochondria were stored at 0°C in 250mM-sucrose, 5mM-Tes (sodium salt), pH7.4. Protein was determined by the biuret method (Gornall *et al.*, 1949).

Methods

pCa_0^{2+} was determined directly in mitochondrial incubations by means of a Ca^{2+}-selective electrode (Radiometer type F2112Ca) with a KCl reference electrode (Radiometer type K801), as previously described (Nicholls, 1978*b*), except that the volume of the incubation chamber was decreased to 0.35ml. The electrode had a linear response to pCa^{2+} (27.8mV/pCa^{2+}) over a range from 4.6 to 6.8 in the presence of nitrilotriacetate as Ca^{2+} chelator. Matrix Ca^{2+} was determined by difference after calculating the total extramitochondrial Ca^{2+} required to achieve the observed pCa_0^{2+} (Nicholls, 1978*b*). Endogenous Ca^{2+} was determined by a Corning Eel model 240 mark II atomic-absorption spectrophotometer, and allowance for this was made in all calculations.

Membrane potential ($\Delta\psi$) was estimated from the Nernst equilibrium of $^{86}Rb^+$ in the presence of valinomycin as previously described (Nicholls, 1974), except that the volume of the samples was decreased to 100μl (Nicholls, 1978*a*).

The relative adenine nucleotide content of the mitochondria was estimated by preincubating the mitochondria in the presence of 0.1μM-[^{3}H]ATP (1.2μCi/ml of incubation) and [^{14}C]sucrose (0.5μCi/ml of incubation) for 2min to allow the specific activities of the ATP, ADP and AMP pools within the matrix to equalize. Additions were then made and samples were filtered at defined times to estimate the net radioactivity counts within the matrix.

Results

When rat liver mitochondria are incubated in the presence of substrate and electroneutrally-permeant weak acid they attain a pCa_0^{2+} that is dependent only on the kinetics of the Ca^{2+} uptake and efflux pathways, and is largely independent of the Ca^{2+} accumulated in the matrix, or the magnitude of $\Delta\psi$ (Nicholls, 1978*b*). However, brain mitochondria incubated under these conditions (Fig. 1, traces *a* and *b*) do not demonstrate this exact buffering of pCa_0^{2+}. Instead, whereas the first portion of Ca^{2+} is taken up until pCa_0^{2+} increases to 6.5, subsequent additions of Ca^{2+} are only accumulated partially, with the result that the steady-state pCa_0^{2+} attained

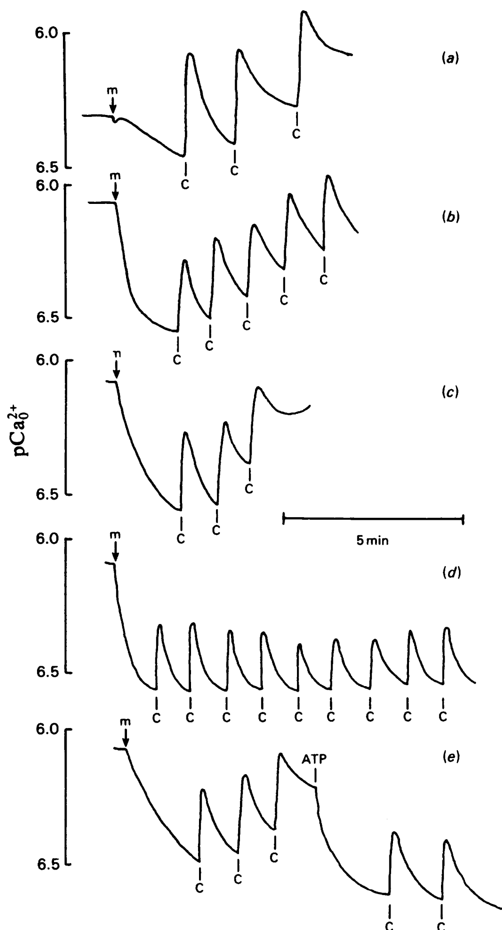

Fig. 1. *The effects of carboxyatractylate, ATP and oligomycin on the steady-state* pCa_0^{2+} *maintained by brain mitochondria*

'Free' brain mitochondria were incubated in a medium containing 75mM-KCl, 10mM-Tes (potassium salt), 2mM-succinate (potassium salt), 2mM-nitrilotriacetate (potassium salt), 14μM-$CaCl_2$, 1μM-rotenone and 16μM-albumin, pH 7.0, 30°C. In trace (*a*), 5mM-acetate (potassium salt) was initially present, the mitochondrial concentration was 1.5mg/ml of incubation, and each addition of Ca^{2+} (C) corresponded to 13nmol/mg of protein. In traces (*b*)–(*e*), 2.3mM-P_i (potassium salt) was initially present, the mitochondrial concentration was 1mg/ml of incubation and each addition of Ca^{2+} (C) corresponded to 25nmol/mg of protein. In trace (*c*) 80μM-carboxyatractylate (potassium salt) was initially present; in trace (*d*) 2μg of oligomycin/ml and 0.2mM-ATP (diethanolammonium salt) were initially present. Addition of mitochondria is indicated by m.

by the mitochondria after each addition decreases. It should be emphasized that this imperfect buffering is first apparent when there is only a slight accumulation of Ca^{2+} within the matrix, and when the free extramitochondrial Ca^{2+} concentration does not increase above 1μM.

These observations are not in accord with the suggestion (Nicholls, 1978*b*,*c*) that Ca^{2+} cycling enables mitochondria to regulate pCa_0^{2+} at a level that is independent of the Ca^{2+} accumulated in the matrix. However the following evidence suggests that superimposed on the Ca^{2+} cycling is the early reversible stage of a progressive damage to mitochondrial integrity.

Atractylate is known to potentiate the loss of Ca^{2+} from the matrix of heart mitochondria (Asimakis & Sordahl, 1977). With the present preparation, both atractylate (results not shown) and carboxyatractylate (Fig. 1*c*) potentiate the inability of the mitochondria to buffer pCa_0^{2+}, such that after three Ca^{2+} additions a net release of Ca^{2+} is observed. In contrast with the effect of these agents, the addition of oligomycin and ATP induces an apparently limitless capacity of brain mitochondria to to restore pCa_0^{2+} to precisely the same value after each addition of Ca^{2+}, until over 200nmol of Ca^{2+}/mg of protein is accumulated in the matrix (Fig. 1*d*). If ATP is added to mitochondria that have accumulated sequential portions of Ca^{2+} in the absence of nucleotide (Fig. 1*e*), additional Ca^{2+} uptake occurs until pCa_0^{2+} is increased to 6.5. Thus no irreversible deterioration of the ability of the incubation to regulate pCa_0^{2+} is apparent under these conditions.

Under the conditions of Fig. 1, in the presence of 2.3mM-P_i, mitochondria incubated in the initial presence of either 80μM-atractylate or 80μM-carboxyatractylate fail to accumulate a single addition of 100nmol of Ca^{2+}/mg of protein, but instead rapidly release the cation after a partial accumulation (results not shown). The addition of 0.2mM-ATP once this release is under way is unable to restore the Ca^{2+}-buffering properties of the mitochondria, indicating that by this stage irreversible effects are apparent. When the atractylate and ATP are both present in the initial incubation the mitochondria retain the subsequent Ca^{2+}-addition, but they fail to do so when carboxyatractylate and ATP are initially present (results not shown). In view of the much greater affinity of carboxyatractylate for the translocator (Klingenberg *et al.*, 1975), these results suggest that a progressive deterioration of the mitochondrial pCa_0^{2+} buffering occurs when the translocator is prevented from binding extra-mitochondrial adenine nucleotides.

Fig. 2 depicts the variation in $\Delta\psi$ as a function of the Ca^{2+} added to brain mitochondria. Portions of Ca^{2+} (25nmol of Ca^{2+}/mg of protein) were added at 1min intervals, and $\Delta\psi$ was determined 55s after each addition, to reproduce the pattern of the experiments depicted in Fig. 1. It is clear that $\Delta\psi$

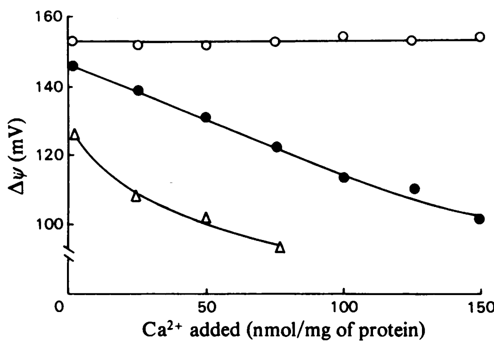

Fig. 2. *The effects of ATP and carboxyatractylate on the maintenance of the membrane potential across the inner membrane of 'free' brain mitochondria during the accumulation of* Ca^{2+}

Mitochondria were incubated (1 mg of protein/ml of incubation) in a medium containing 75 mM-NaCl, 10 mM-Tes (sodium salt), 2 mM-succinate (sodium salt), 2 mM-nitrilotriacetate (sodium salt), 2.3 mM-phosphate (sodium salt), 0.1 mM-KCl, 1 μM-rotenone, 16 μM-albumin, 0.5 μM-valinomycin, 50 μM-^{86}RbCl (0.1 μCi/ml) and $^{3}H_2O$ (1.2 μCi/ml). Portions of $^{45}CaCl_2$ (25 nmol/mg of mitochondrial protein, 0.05 μCi/ml) were added to the incubation 2 min after addition of mitochondria and at each subsequent minute. Mitochondria were separated for the determination of $\Delta\psi$ 55 s after each addition of Ca^{2+}. Symbols: ○, 200 μM-ATP and 2 μg of oligomycin/ml initially present; ●, control; △, 80 μM-carboxyatractylate initially present.

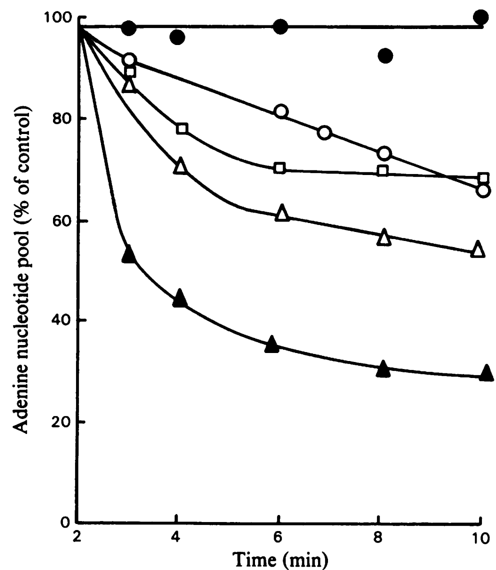

Fig. 3. *The effect of ATP,* Ca^{2+} *and carboxyatractylate on the maintenance of the adenine nucleotide pool within the matrix of 'free' brain mitochondria*

Mitochondria (1 mg of protein/ml of incubation) were incubated in a medium containing 75 mM-NaCl, 10 mM-Tes (sodium salt), 2 mM-succinate (sodium salt), 2 mM-nitrilotriacetate (sodium salt), 1 μM-rotenone, 2 mM-phosphate (sodium salt), 16 μM-albumin, 50 μM-EGTA (sodium salt), 0.1 mM-KCl, 0.5 μM-valinomycin, 50 μM-^{86}RbCl (0.1 μCi/ml) and [^{14}C]sucrose (0.5 μCi/ml). 0.1 μM-[^{3}H]ATP (1.2 μCi/ml) was initially present, except in (○), where the [^{3}H]ATP was 50 μM. Mitochondria were separated at defined times, and the total ^{3}H-labelled adenine nucleotide pool associated with the mitochondria was determined. Symbols: ○, control; □, 80 μM-carboxyatractylate added at 2 min; △, 150 μM-$CaCl_2$ added at 2 min; ▲, 150 μM-$CaCl_2$ and 80 μM-carboxyatractylate added at 2 min; ●, control, 50 μM-[^{3}H]ATP present from start.

varies in parallel with the observed changes in the steady-state pCa_0^{2+}. Under control conditions there is a steady decrease in $\Delta\psi$ as Ca^{2+} is accumulated, even though 2.3 mM-P_i is present, and this correlates with the decreased pCa_0^{2+} (Fig. 1*b*). The decrease in $\Delta\psi$ is greatly potentiated by carboxyatractylate, and is completely prevented by the presence of ATP and oligomycin. The imperfect buffering of Ca^{2+} can thus be explained adequately as a consequence of a decrease in $\Delta\psi$ to the extent that thermodynamic reversibility of the uniporter can occur (Nicholls, 1978*b*).

Many conditions that lead to the irreversible loss of Ca^{2+} from mitochondria are associated with a net decrease in the adenine nucleotide content of the mitochondria matrix (Ernster, 1956; Meisner & Klingenberg, 1968; Out *et al.*, 1971; Sul *et al.*, 1976; Prpić *et al.*, 1978). Fig. 3 demonstrates that a similar explanation can account for the spontaneous and carboxyatractylate-enhanced decrease in $\Delta\psi$. It is significant that even in the virtual absence of Ca^{2+} (i.e. in the presence of 50 μM-EGTA), there is a slow depletion of matrix adenine nucleotides. Ca^{2+} enhanced the depletion, and whereas carboxyatractylate alone has little effect, the cation and inhibitor together produce maximal depletion. ATP (50 μM) is sufficient to prevent detectable loss of nucleotides. In contrast with previous reports with liver mitochondria (Carafoli *et al.*, 1965), no increase in matrix adenine nucleotide content could be detected in the presence of ATP.

It is thus apparent that brain mitochondria incubated in the presence of millimolar concentrations of P_i are unstable unless a low concentration of ATP is present in the medium to prevent the unidirectional loss of adenine nucleotides from the matrix, with the consequent loss in the ability of the mitochondria to maintain a high membrane potential.

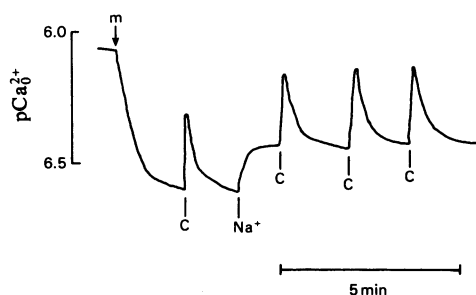

Fig. 4. *The effect of Na^+ on the set-point for the buffering of pCa_0^{2+} by 'free' brain mitochondria*
Mitochondria (1 mg of protein/ml of incubation) were incubated under the conditions described in the legend to Fig. 1 with the addition of 200 μM-ATP, 2 μg of oligomycin/mg of protein and 2.3 mM-P_i (potassium salt). Where indicated 10 mM-NaCl was added. Each arrow represents the addition of 25 nmol of Ca^{2+} (C)/mg of mitochondrial protein. m indicates the addition of mitochondria.

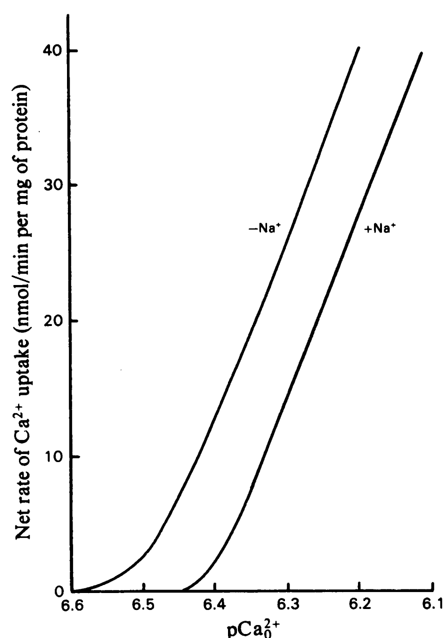

Fig. 5. *The effect of Na^+ on the net rate of uptake of Ca^{2+} by 'free' brain mitochondria as a function of pCa_0^{2+}*
Mitochondria (1 mg of protein/ml of incubation) were incubated in the basic medium described in the legend to Fig. 1 with the addition of 200 μM-ATP, 2 μg of oligomycin/mg of protein and 2.3 mM-P_i (potassium salt). After preincubating for 2 min, 57 nmol of Ca^{2+}/mg of mitochondrial protein was added, and the rate of Ca^{2+} uptake as a function of pCa_0^{2+} was calculated from the Ca^{2+} electrode trace. In the lower trace 10 mM-NaCl was initially present.

The efficient buffering of the steady-state pCa_0^{2+} in the presence of ATP and oligomycin is in itself indicative of an independent efflux pathway operating under these conditions (Nicholls, 1978*b*,*c*). In the case of liver mitochondria (Nicholls, 1978*b*) pCa_0^{2+} stabilizes at a value that allows the Ca^{2+} uniport (whose activity is highly dependent on the concentrations of Ca^{2+} in the medium) to balance the activity of the efflux pathway (which in contrast appears to be independent of the concentration of Ca^{2+} in the matrix). It would thus be predicted for brain mitochondria that activation of the efflux pathway would lead to a net efflux of Ca^{2+} from the matrix until pCa_0^{2+} decreased sufficiently to enable the uniporter to match the increased absolute efflux rate. As shown in Fig. 4, addition of 10 mM-NaCl in the presence of ATP and oligomycin results in a net Ca^{2+} efflux until pCa_0^{2+} has decreased by 0.2 units and that, as predicted, subsequent additions of Ca^{2+} are precisely buffered.

To test whether the Na^+-induced decrease in the pCa_0^{2+} maintained by the brain mitochondria can be quantitatively accounted for by activation of the efflux pathway, the experiment depicted in Fig. 5 was performed. The net rate of Ca^{2+} uptake into the matrix in the presence and absence of 10 mM-NaCl was computed from Ca^{2+}-electrode traces, and plotted as a function of pCa_0^{2+}. As was observed for liver mitochondria (Nicholls, 1978*b*), the net uptake rate increases very rapidly as pCa_0^{2+} decreases. At $pCa_0^{2+} = 6.2$ (0.6 μM free Ca^{2+}) the net uptake rate in the absence of Na^+ reaches 40 nmol of Ca^{2+}/min per mg of protein. The net uptake rate at any pCa_0^{2+} in the presence of Na^+ is about 10 nmol of Ca^{2+}/min per mg of protein less than the corresponding rate in the absence of Na^+. The absolute efflux rates determined in the absence of ATP or oligomycin after addition of Ruthenium Red are respectively 11.5 and 1.3 nmol of Ca^{2+}/min per mg of protein in the presence and absence of Na^+ (Nicholls, 1978*a*). It is therefore clear that the effect of Na^+ on Ca^{2+} cycling in the presence of ATP and oligomycin can be precisely accounted for by the activation of the efflux pathway.

Discussion

Low concentrations of ATP, ADP or oligomycin can prevent the irreversible damage associated with the presence of P_i (see Lehninger *et al.*, 1967; Bygrave, 1978; Carafoli & Crompton, 1978). Indeed mitochondria are capable of surviving additions of millimolar free Ca^{2+}, resulting in the so-called

'massive-loading' of the matrix with in excess of 1 μmol of Ca^{2+}/mg of protein (see Lehninger *et al.*, 1967). Because of the high Ca^{2+} concentrations used in massive-loading experiments, and because of the finding that added adenine nucleotides were not required for limited Ca^{2+} accumulation (Rossi & Lehninger, 1964), there has been a consensus that physiologically significant aspects of mitochondrial Ca^{2+} transport should be observable in the absence of added nucleotide (Lehninger *et al.*, 1967). However, the present paper demonstrates that, at least for brain mitochondria, adenine nucleotides are required to prevent misleading artefacts, even when a very limited accumulation of Ca^{2+} takes place from media containing sub-micromolar free Ca^{2+} concentrations.

Kinetic and membrane potential-dependent efflux pathways.

Our observation that the Na^+-activated efflux pathway remains functional in the presence of ATP and oligomycin is in contrast with the proposal of Harris (1977, 1979), who reported that the Na^+-induced net efflux of Ca^{2+} from heart mitochondria was greatly diminished by the presence of ATP in the incubation medium. However, in the presence of ATP this author observed that the addition of Na^+ increased the steady-state free Ca^{2+} by almost 1 μM (Fig. 8*e* of Harris, 1977). We suggest that the reason that omission of ATP appears to further enhance the Na^+-induced efflux could be the superimposition of a time-dependent efflux caused by a decreasing membrane potential. Similarly, the apparent ability of oligomycin to reverse Na^+-dependent Ca^{2+} efflux from heart mitochondria (Fig. 8*d* of Harris, 1977) could be reconciled with our results if the heart mitochondria were operating with a membrane potential somewhat lower than 120 mV. Thus, on addition of Na^+ both kinetic and membrane-potential-dependent efflux mechanisms would be operative. Oligomycin could act not by inhibiting an independent efflux pathway, but by increasing $\Delta\psi$ and preventing reversal of the uniport, in the same way that ATP can cause an increased Ca^{2+} uptake in the absence of Na^+ in the present preparation (Fig. 1*e*).

A second case where, we propose, kinetic and membrane-potential-dependent efflux mechanisms may require re-evaluation is in the time-dependent net Ca^{2+} efflux from liver mitochondria, which is observed in the presence of *N*-ethylmaleimide (Ramachandran & Bygrave, 1978). These authors demonstrate that the net Ca^{2+} efflux under these conditions is not a consequence of gross mitochondrial damage, as the proton electrochemical gradient is maintained in the region of 200 mV (but see Pfeiffer *et al.*, 1978). The immediate effect of Ca^{2+} uptake in the presence of *N*-ethylmaleimide is to greatly enhance ΔpH and decrease $\Delta\psi$ (see also Nicholls, 1978*b*). However, during the subsequent time-dependent loss of Ca^{2+} from the matrix, $\Delta\psi$ continues to decrease, from 106 mV to 92 mV (Fig. 3 of Ramachandran & Bygrave, 1978). These membrane potentials are within the range over which Ca^{2+} distribution across the membrane approximates to a thermodynamic equilibrium for the Ca^{2+} uniporter (Rottenberg & Scarpa, 1974; Nicholls, 1978*b*). In this case the observed decrease in $\Delta\psi$ would decrease the gradient of Ca^{2+} across the membrane by about 3-fold, which is what is in fact observed (Fig. 3 of Ramachandran & Bygrave, 1978). We therefore propose that the *N*-ethylmaleimide-induced Ca^{2+} efflux provides no evidence for the existence of Ca^{2+} cycling, but instead reflects a thermodynamic reversal of the uniporter.

A third possible example of membrane-potential-dependent efflux is that potentiated by phosphoenolpyruvate in the presence of P_i (Chudapongse & Haugaard, 1973; Peng *et al.*, 1974; Sul *et al.*, 1976). Thus Roos *et al.* (1978) have clearly demonstrated that the net Ca^{2+} efflux observed under these conditions is a consequence of a decreased membrane potential.

It is clear from these examples, and from the present paper, that unless a Ca^{2+} efflux can be shown to operate in the presence of a high membrane potential it cannot be assigned unequivocally to an independent efflux pathway. So far, only the steady-state efflux described for liver mitochondria (Puskin *et al.*, 1976; Azzone *et al.*, 1977; Nicholls, 1978*b*; Åkerman, 1978) or for heart and brain mitochondria (Crompton *et al.*, 1976, 1978; Nicholls, 1978*a*) satisfies this criterion.

The capacity of brain mitochondria to regulate pCa_0^{2+}

The ability of brain mitochondria to buffer and regulate pCa_0^{2+} in the presence of ATP and P_i is impressive not only because of the capacity of their matrices for Ca^{2+} (Fig. 1), but also for their ability to decrease the extramitochondrial free Ca^{2+} to less than 0.3 μM; to respond with extreme rapidity to a slight perturbation in pCa_0^{2+}; and to modulate the set-point at which pCa_0^{2+} is buffered. Brain mitochondria are thus well adapted for a putative role in the regulation of cytosolic free Ca^{2+}.

Recently it has been demonstrated that vesicular preparations from isolated nerve endings possess Ca^{2+}-activated ATPase activity, together with the ability to accumulate Ca^{2+}, and it has been suggested that these vesicles, rather than the mitochondria contained within the nerve endings, play the major role in the regulation of cytosolic Ca^{2+} (Rahamimoff & Abramovitz, 1978*a*,*b*; Blaustein *et al.*, 1978). However, Ca^{2+}-uptake rates by these preparations from media containing 0.5–1 μM-Ca^{2+}

are less than 0.2 nmol of Ca^{2+}/min per mg of particle protein, or less than 1% of the corresponding mitochondrial rates (Fig. 5). Additionally, the capacity of the preparations to accumulate Ca^{2+} is very limited.

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