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The Respiration of Rat Brain Mitochondria Purified by Phase Partition. Effects of K^+, Mg^{2+} and Ca^{2+}

J. Sánchez-Prieto and M. J. López-Pérez*

Departamento de Bioquímica
Facultad de Farmacia
Universidad Complutense
28040 Madrid

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J. SANCHEZ-PRIETO and M. J. LOPEZ-PEREZ. *The respiration of rat brain mitochondria purified by phase partition. Effects of K^+, Mg^{2+} and Ca^{2+}.* Rev. esp. Fisiol., **42** (4), 427-434, 1986.

Highly purified brain mitochondria have been prepared by Na^+, NH_4^+ or K^+-containing two-phase systems. K^+ stimulated the basal rate of respiration in the three mitochondrial preparations. However, K^+ only stimulated the maximal oxidation rate (state 3 respitation rates) in those mitochondria prepared by K^+-free (Na^+ or NH_4^+-containing) two-phase systems. The increase in the basal rates of respiration induced by exogenous K^+ correlates with the mitochondrial swelling rates. The stimulatory effect of K^+ on maximal oxidation rates seems to reflect the K^+ depletion of brain mitochondria when prepared by K^+-free procedures.

Key words: Mitochondrial respiration, K^+, Mg^{2+}, Ca^{2+}.

Stimulation of respiration of brain mitochondria by K^+ has been reported by several laboratories (3, 7, 18, 19, 26). However, the nature of this enhancement has been the subject of some controversy. CLARK and NICKLAS (7), using a relatively uncontaminated rat brain mitochondria preparation have reported that exogenous K^+ was required for maximal oxidation rates with various substrates. Whereas in other studies (3), K^+ only stimulated the oxidation of pyruvate, highly purified rat brain mitochondria have been recently obtained by K^+-containing two-phase systems (15). Mitochondria prepared by this procedure showed a good respiratory control but they were insensitive to the stimulation of the maximal oxidation rates (state 3 rates) by exogenous K^+.

In the present work rat brain mitochondria in K^+-free two-phase systems (NH_4^+ or Na^+-containing two—phase systems) have been prepared, and the effect of exogenous K^+ on respiration with various substrates

* . To whom correspondence should be addressed. Departamento de Bioquímica. Facultad de Veterinaria. Universidad de Zaragoza. 50013 Zaragoza (Spain).

has been studied. The results indicate that there are two phenomena associated with the stimulatory effect of K^+ on mitochondrial respiration: first a K^+ stimulation of the basal rates of respiration and secondly a K^+ stimulation of maximal respiration rates which is only observed in mitochondria prepared in K^+-free two-phase systems. The evidence suggests that this K^+ stimulation might reflect a K^+ depletion of brain mitochondria prepared by K^+-free procedures.

Materials and Methods

Preparation of mitochondria. Except for the salt composition of medium B and phase mixture, the method for mitochondrial preparation was as previously described (15). «Crude mitochondria» obtained by centrifugation on 6 % Ficoll were diluted with 20 ml of medium B containing: 0.32 M mannitol, 0.1 mM EDTA and 5 mM potassium phosphate buffer pH 7.8. «K^+-containing medium B», or 5 mM sodium phosphate buffer pH 7.8. «Na^+-containing medium B», or 5 mM ammonium hydroxide-phosphoric acid buffer pH 7.8. «NH_4^+-containing medium B», Suspensions were centrifuged at 19,000 x *g* for 20 min and the pellets resuspended to 1 ml with K^+, Na^+ or NH_4^+-containing medium B. Then, 1 g of mitochondrial suspension was added to 7 g fo K^+, Na^+ or NH_4^+-containing two-phase systems. The two-phase systems were prepared as previously described (15) using 5 mM potassium phosphate, «K^+-containing two-phase systems» or 5 mM sodium phosphate, «Na^+-containing two-phase systems» or 5 mM ammonium hydroxide-phosphate, «Na^+-containing two-phase systems» or 5 mM ammonium hydroxide-ammonium chloride was added to the phase system to adjust the partition of mitochondria to the lower phase as it will be discussed later.

Assay procedures. Lactate dehydrogenase was assayed according to the method of CLARK and NICKLAS (7), fumarase according to RACKER (22), cytochrome c oxidase according to the method of TURNER (25) and acetylcholinesterase according to the method of ELLMAN *et al.* (8). Mitochondrial protein was determined by the biuret method (11) using bovine serum albumin as standard.

Mitochondrial respiration was measured polarographically at 25°C in an incubation medium with a total volume of 1 ml consisting of either 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 10 mM phosphate/Tris, 10 mM Tris/HCl, 0.05 mM EDTA, pH 7.4 (100 mM K^+ medium) or 5 mM KCl, 225 mM mannitol, 75 mM sucrose, 10 mM phosphate/Tris, 10 mM Tris/HCl, 0.05 mM EDTA, final pH 7.4 (5 mM K^+ medium). Mitochondrial protein (0.75-1 mg), together with the appropriate substrates were added to the respiration medium with a final volume of 1 ml. The substrate solutions were prepared with the acid forms and neutralized with Tris solution till pH 7. Respiration was stimulated by the addition of either ADP (state 3) (6) or the uncoupler FCCP.*

Mitochondrial swelling was monitored by absorbance change at 600 nm with a Unicam SP-1800 spectrophotometer.

Total potassium content of mitochondrial preparations was assayed by atomic absorption spectroscopy.

For the measurement of mitochondrial volumes mitochondria (approx. 4 mg/ml) prepared either in NH_4^+ or K^+-containing two-phase systems were incubated at 0°C during 5 min in medium B. ^3H_2O and (carboxyl-^{14}C) inulin were present at 2 and 0.6 μCi/ml, respectively. Sedimentation of mitochondria was performed in 1.5 ml Ep-

* *Abbreviations.* MOPS, 3-(N-morpholino) propanesulfonic acid. FCCP, carbonylcyanide p-trifluorometoxiphenylhydrazone. $\Delta\mu H^+$, electrochemical gradient of protons.

pendorf tubes containing from the bottom: 0.25 ml of 14 % perchloric acid, 0.5 ml of silicone oil mixture and 0.4 ml of NH_4^+ or K^+-containing medium B. A 0.3 ml aliquot from the incubation was rapidly mixed with the top layer and centrifuged at 10,000 x *g* for 1 min in a Eppendorf microfuge. A sample from the top layer was with-drawn and the silicone aspirated. A 0.2 ml aliquot of the perchloric acid layer was neutralized with 2 M KOH-0.3 M MOPS. Aliquots from the top layer and from the neutralized perchloric acid layer were taken for ^3H and ^{14}C assay using a liquid scintillation counter.

Results and Discussion

It is well established that the salt composition determines the electrostatic potential between the phases of aqueous dextran-poly (ethylene glycol) two-phase systems (1, 14). The different distribution of phosphate anions in the phase generates an interfacial potential introducing a positive charge in the upper poly (ethylene glycol) phase with respect to the lower dextran phase. This interfacial potential is higher in the case of ammonium phosphate than for the sodium or potassium salts (9). The use of sodium phosphate instead of potassium phosphate in the two-phase systems did not modify the partition of the mitochondria (data not shown). However, the partition of this particle into the dextran lower phase was significantly decreased when the two-phase systems were prepared with 5 mM ammonium hydroxide-phosphoric acid buffer instead of 5 mM potassium phosphate buffer. In the latter case, the addition of 5 mM ammonium chloride, which generates a negative interfacial potential (9), compensated for the effect of the ammonium phosphate and adjusted the partition of the mitochondria to the dextran lower phase.

Mitochondria isolated by Na^+ or NH_4^+-containing two-phase systems showed an excellent degree of purity (table I), as deduced by the low activities of lactate dehydrogenase and acetylcholinesterase, markers of synaptosomal contamination. The high activities of the mitochondrial enzymes fumarase and cytochrome c oxidase were also in agreement with the results already found in mitochondria prepared by K^+-containing two-phase systems (15). These results corroborate the advantage of phase partition methods over centrifugation methods in the purification of brain mitochondria. The differences could be due to the fact that centrifugation methods separate cell particles according to size and density, whereas phase partition methods separate organelles according to the surface properties of their membranes, such as the lipidic content (23, 24).

The respiratory activities of mitochondria prepared by K^+, Na^+ or NH_4^+-contain-

Table I. *Specific enzyme activities of mitochondria isolated by Na^+ or NH_4^+-containing two-phase systems.*

Enzyme activities are expressed as nmol × min^{-1} × mg^{-1}. Values are the average ± S.D. of three separate mitochondrial preparations.

Preparation	Lactate dehydrogenase		Acetyl-cholinesterase	Cytochrome c oxidase	Fumarase
	— Triton	+ Triton			
Na^+-containing two-phase systems	3 ± 1	19 ± 3	5 ± 1	468 ± 43	302 ± 16
NH_4^+-containing two-phase systems	1 ± 0	7 ± 1	4 ± 1	501 ± 70	454 ± 100

Table II. *Respiratory activities of mitochondria isolated by K^+, Na^+ or NH_4^+-containing two-phase systems.*

Respiration media containing 5 or 100 mM KCl were used (see Materials and Methods). The composition of media with 0 mM KCl was similar to that in medium containing 5 mM KCl where choline chloride instead of KCl was used. Respiration rates expressed as natoms of oxygen × min^{-1} × mg^{-1} of protein. State 3 rates were induced by addition of 0.2 mM ADP. RCR is the quotient between state 3 and state 4 rates. The values are the means ± S.D. of three separate mitochondrial preparations.

Preparation	KCl (mM)	2.5 mM malate+5 mM glutamate			10 mM succinate			2.5 mM malate+5 mM pyruvate		
		Basal	State 3	RCR	Basal	State 3	RCR	Basal	State 3	RCR
K^+-containing two-phase systems	5	15±3	85±3	7.0	23±2	103±4	5.1	16±5	73±4	4.8
	100	31±2	87±5	3.7	37±5	95±6	2.2	32±2	83±2	2.4
Na^+-containing two-phase systems	5	14±6	77±3	4.8	32±3	114±24	3.9	21±4	82±18	3.9
	100	28±6	106±5	3.5	42±5	143±14	2.6	46±1	109±7	2.4
NH_4^+-containing two-phase systems	0	13±3	58±2	4.8	27±6	101±7	4.2	20±6	74±1	4.1
	5	18±2	63±20	5.2	30±10	112±19	4.1	20±3	74±12	4.3
	100	36±3	87±10	2.7	54±12	149±10	2.5	60±20	134±18	2.1

ing two-phase systems are shown in table II. The increase in the concentration of exogenous K^+ from 5 to 100 mM induced a stimulation of the basal rates of respiration with the three mitochondrial preparations. This uncoupling effect was obtained with all the three substrates assayed. Mitochondria prepared by Na^+ or NH_4^+-containing two-phase systems showed an increase in the state 3 rates of respiration by high K^+ concentration in the medium. Conversely, mitochondria isolated by K^+-containing two-phase systems did not present this stimulatory effect as previously reported (15). The respiratory control ratios (RCR's) were higher at 5 mM K^+ (3.9-7) than at 100 mM K^+ (2.1-3.7). P/O quotients with pyruvate (+ malate) and glutamate (+ malate) as substrates were 2.6-2.9 and 1.7-1.9 with succinate (data not shown) proving the intactness of the mitochondrial preparations obtained.

K^+ stimulation of brain mitochondria respiration seems to be a consequence of two distinctive phenomena: an uncoupling effect by increase of the basal rates of respiration and increase of the maximal respiration rates. To shed light on the mechanism involved in K^+ influences upon respiration, mitochondria were tested for K^+ permeability by measuring the energy-dependent swelling at different concentrations of the cation.

Table III. *Swelling rates of mitochondria isolated by K^+, Na^+ or NH_4^+-containing two-phase systems.*

Swelling experiments were carried out in the respiration media at different concentration of K^+. 2.5 mM malate plus 5 mM glutamate were present as substrates. Swelling rates were calculated from the slope of absorbance decrease 30 s after addition of mitochondria and expressed as the change × min^{-1} in the percent of initial absorbance at 600 nm (Δ % A_i 600 × min^{-1}). Values are the average ± S.D. of three separate mitochondrial preparations.

Preparation	KCl (mM)	Swelling rate (Δ % A_i 600 × min^{-1})
K^+-containing two-phase systems	5	2.1 ± 0.3
	100	5.6 ± 0.7
Na^+-containing two-phase systems	5	1.7 ± 0.2
	100	4.0 ± 0.3
NH_4^+-containing two-phase systems	5	2.4 ± 0.3
	100	5.5 ± 0.4

Figure 1 shows that the swelling rates of energized mitochondria are dependent of the K^+ concentration in the medium. Valinomycin, a K^+-conducting ionophore (17) when added at concentrations that saturates the transport of K^+ accelerates five times the swelling rate at 100 mM KCl. The swelling rate of mitochondria prepared by K^+, Na^+ or NH_4^+-containing two-phase systems were about twice higher in 100 mM KCl than in 5 mM KCl media (table III). The good correlations observed between the increase in the swelling rate at high concentrations of K^+ (table III), and the stimulation induced by this cation on the basal rates of respiration (table II) should be noted.

These results suggest that the uncoupling effect caused by K^+ seems to be a consequence of the electrophoretic movement of K^+ into mitochondria in response to the negative membrane potential of the energized organelle. This entry of K^+ into mitochondria could decrease the $\Delta\mu H^+$ as postulated by MITCHELL (16) by exchange between intramitochondrial K^+ and H^+, via a K^+/H^+ antiporter (5, 10).

The K^+ stimulation of maximal respiration rates seems to depend on the presence of this cation in the isolation media, since this stimulatory effect only occurs when mitochondria are prepared in K^+-free two-phase systems. In order to test if this stimulatory effect was related to the K^+ content of the mitochondrial preparations, the total K^+ content of mitochondria prepared by K^+, Na^+ or NH_4^+-containing two-phase systems was assayed. Table IV shows that mitochondria prepared in K^+-containing media retain about ten times more K^+ than mitochondria prepared in K^+-free media (Na^+ or NH_4^+-containing two-phase systems). These results suggest that the effect of K^+ on maximal oxidation rates might reflect a K^+ depletion of mitochondria when prepared in K^+-free media. In this regard, BERNARD and COCKRELL (3), have shown that mitochondria prepared by a K^+-containing procedure retain a high K^+ content (230 natoms $\times$ mg^{-1} of protein) and their maximal oxidation rates were insensitive to K^+. On the other hand, mito-

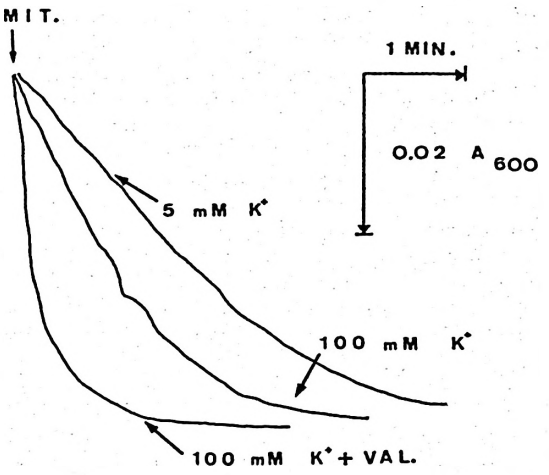

Fig. 1. *Mitochondrial swelling at different K^+ concentrations.*
Mitochondria (0.4 mg/ml) isolated by NH^+_4-containing two-phase systems were added to the respiration media containing 5 or 100 mM K^+ in the presence of 5 mM glutamate + 2.5 mM malate as substrates. The decrease in absorbance (indicating mitochondrial swelling) was recorded continuously at 600 nm. When indicated valinomycin (20 ng $\times$ mg^{-1} of protein) was present.

Table IV. *Potassium content of mitochondria isolated by K^+, Na^+ or NH_4^+-containing two-phase systems.*
Mitochondria prepared by K^+, Na^+ or NH_4^+-containing two-phase systems were suspended in NH_4^+-containing medium B and the K^+ content assayed. Values are the average ± S.D. of three determinations.

Preparation	nmoles of $K^+ \times mg^{-1}$ protein
K^+-containing two-phase systems	136.6 ± 5.1
Na^+-containing two-phase systems	14.1 ± 0.1
NH_4^+-containing two-phase systems	16.2 ± 0.9

Table V. *Potassium effect on the maximal respiration rates of mitochondria in the presence of an uncoupler.*

Mitochondria isolated in NH_4^+-containing two-phase systems were used. Respiration was carried out in 5 or 100 mM KCl containing media in the presence of 5 mM pyruvate plus 2.5 mM malate as substrates. Respiration rates are expressed as natoms of oxygen × min^{-1} × mg^{-1} of protein. State 3 rates were induced with 0.2 mM ADP or 0.5 μM FCCP. Values are the average ± S.D. of three determinations.

KCl (mM)	Basal rate	State 3		RCR
5	23 ± 3	100 ± 4	(ADP)	4.1
		109 ± 7	(FCCP)	—
100	77 ± 10	145 ± 5	(ADP)	1.7
		154 ± 10	(FCCP)	—

chondria prepared by K-free procedures (7, 12, 13) were stimulated by K^+, when their respiratory activity was assayed in the presence of ADP with several substrates, although in these cases the authors did not give the K^+ content of their mitochondria preparations.

In order to study whether the different K^+ content of mitochondria prepared either by K^+-free or K^+-containing two-phase systems were responsible for a different size of isolated mitochondria, the volume of the mitochondrial preparations was measured by incubating the organelles with ^3H_2O and ^{14}C (carboxyl) inulin. The results showed no differences in the volume of mitochondria prepared either by K^+ or NH_4^+-containing two-phase systems with values (μl × mg^{-1} of protein ± S.D. (n) No. of determinations) of 0.54 ± 0.09 (n = 4) and 0.55 ± 0.07 (n = 4) respectively.

It has been suggested by CLARK and NICKLAS (7) that the stimulation by K^+ of the state 3 rates of respiration in brain mitochondria might be related to an increase in the availability of substrates for oxidation. The results shown in table V make unlikely this suggestion since K^+ stimulated the oxygen uptake of mitochondria respiring in the presence of pyruvate, a substrate

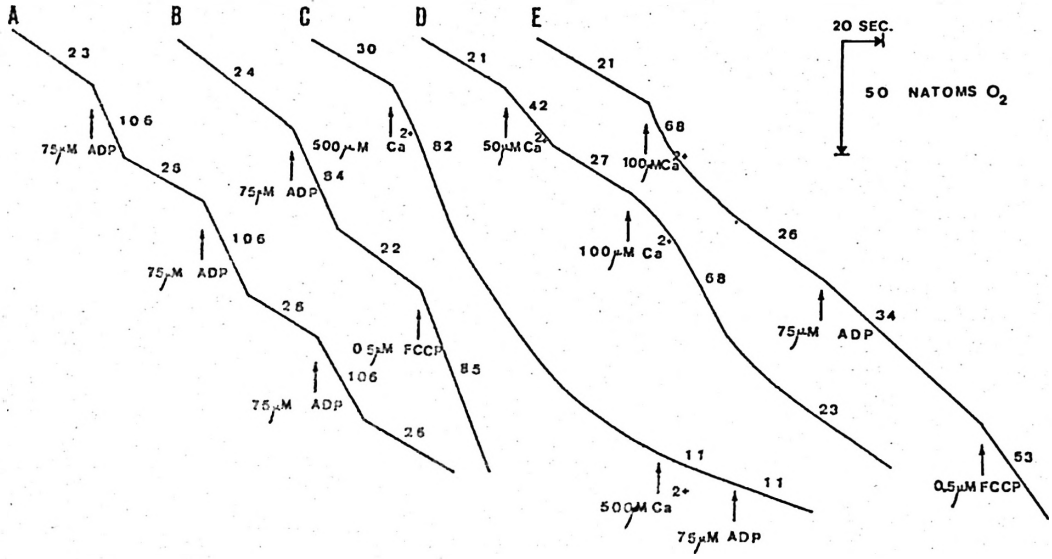

Fig. 2. *Ca^{2+} effects on respiration of brain mitochondria.*
Mitochondria isolated by NH_4^+-containing two-phase systems were used. Respiration activity was measured in media containing 5 mM K^+ with 5 mM glutamate plus 2.5 mM malate as substrates. Figures are natoms of oxygen × min^{-1} × mg of protein.

Table VI. *Effects of Mg^{2+} on mitochondrial respiration.*
Mitochondria isolated in NH_4^+-containing two-phase systems were used. Respiration media in the presence of 5 mM glutamate plus 2.5 mM malate as substrate were used. Other experimental conditions as in table V.

KCl (mM)	Basal rate	State 3	RCR
5	28 ± 7	130 ± 10 (ADP)	5.2
100	55 ± 15	164 ± 10 (ADP)	3.1
100+5 mM MgCl	38 ± 4	184 ± 20 (ADP)	6.8
100+5 mM MgCl	39 ± 7	186 ± 13 (FCCP)	—

known to be transported into mitochondria by a proton symport system (2, 20, 21), even in the presence of FCCP, a H^+ ionophore which decreases both the electrical and chemical components of the $\Delta\mu H^+$. It appears more likely that the K^+ stimulation on maximal respiration rates of brain mitochondria could be due to changes in the electron transport chain as a consequence of the K^+ depletion that occurs when mitochondria are prepared by K^+-free procedures.

The addition of Mg^{2+} to brain mitochondria respiring in high K^+ concentrations produces a significant augmentation of the RCR by decreasing the basal rate and increasing the state 3 rates of respiration (table VI). The decrease in respiration produced by Mg^{2+} is opposed to the uncoupling effect produced by high K^+ concentrations and could be explained as an inhibitory effect of Mg^{2+} on the permeability to K^+ of mitochondrial membrane. In this regard, WEHRLE *et al.* (28) have shown that the removal of Mg^{2+}-membrane bound, induced an increase in the permeability to K^+ in liver and heart mitochondria. Similar results have been observed by BERNARD and COCKRELL (4) in a preparation of rat brain mitochondria with some contamination by synaptosomes. The mechanism by which Mg^{2+} + could interact with the K^+ permeability of mitochondria is by blocking the K^+/H^+ exchanger present in the inner mitochondrial membrane (5, 10).

The addition of Ca^{2+} to a well coupled mitochondrial preparation, figure 2 A and B, induced first an increase and then a decrease in the oxygen uptake, with lack of the subsequent transition to state 3 by addition of ADP. This damage caused by Ca^{2+} on mitochondrial oxidative phosphorylation could be interpreted as an effect of the cation on the electron transport chain. However, the results shown in figure 2 E, suggest that Ca^{2+} could also interact with the transport of ADP into mitochondria, since after the addition of the cation there is still some gradient of H^+ which is not used to phosphorylate ADP. In this regard, it has been suggested that mitochondrial ATP might co-precipitate with the calcium phosphate making difficult the exchange between ADP an ATP (27). An alternative explanation for the lack of phosphorylation capacity of mitochondria in the presence of calcium is an inhibitory effect of this cation on mitochondrial F_1-ATPase. In conclusion, whatever the interaction mechanisms between Ca^{2+} and oxidative phosphorylation are, these experiments clearly show that the presence of Ca^{2+} should be avoided from the isolation media in order to preserve the metabolic integrity of brain mitochondria.

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Resumen

Se estudia el efecto del K sobre mitocondrias de cerebro de rata altamente purificadas con sistemas bifásicos que contienen Na^+, NH_4^+ o K^+. El K^+ estimula la respiración basal de las tres preparaciones mitocondriales, en cambio, sólo se incrementa el estado 3 de respiración en las preparaciones mitocondriales obtenidas en su ausencia. El incremento de la respiración basal se corresponde con el hinchamiento mitocondrial inducido por el K^+. Sobre el consumo de oxígeno del estado 3, parece reflejar la depleción de K^+ de las mitocondrias de cerebro cuando se preparan en medios que no lo contienen.

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