Biochimica et Biophysica Acta, 548 (1979) 173—186

BBA 47741

THE RESPIRATION OF BRAIN MITOCHONDRIA AND ITS REGULATION BY MONOVALENT CATION TRANSPORT

PHYLLIS A. BERNARD and RONALD S. COCKRELL *

Edward A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, MO 63104 (U.S.A.)

(Received April 11th, 1979)

Key words: Mitochondrial respiration regulation; Cation transport; (Rat brain)

Summary

The Na^+ and K^+ permeability properties of rat brain mitochondria were determined to explain the influences of these cations upon respiration. A new procedure for isolating exceptionally intact mitochondria with minimal contamination by synaptosomes was developed for this purpose.

Respiration was uncoupled by Na^+ and less so by K^+. Uncoupling was maximal in the presence of EDTA plus P_i and was decreased by Mg^{2+}. Maximal uncoupler-stimulated respiration rates were inhibited by Na^+ but largely unaffected by K^+. The inhibition by Na^+ was relatively insensitive to Mg^{2+}. Membrane Na^+ and K^+ conductances as well as neutral exchanges (Na^+/H^+ and K^+/H^+ antiport activities) were determined by swelling measurements and correlated with metabolic effects of the cations.

Cation conductance, i.e. electrophoretic Na^+ or K^+ permeation, was increased by EDTA ($Na^+ > K^+$) and decreased by Mg^{2+}. Magnesium preferentially suppressed Na^+ conductance so as to reverse the cation selectivity ($K^+ > Na^+$). Neutral cation/H^+ exchange rates ($Na^+ > K^+$) were not influenced by chelator or Mg^{2+}.

The extent of cation-dependent uncoupling of respiration correlated best with the inner membrane conductance of the ion according to an empirical relationship derived with the model K^+ conductor valinomycin. The metabolic influences of Na^+ and K^+ can be explained in terms of coupled flow of these ions with protons and their effect upon the H^+ electrochemical gradient although alternative possibilities are discussed. These in vitro studies are compared to previous observations in situ to assess their physiological significance.

* To whom correspondence should be sent.
Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Introduction

Various effects of monovalent cations upon the respiration of brain slices and homogenates have been reported which are not observed with other tissues [1—11]. Potassium accelerates the respiration of brain slices [1—3] although Na^+ is required [1,4—6]. This suggests K^+ activation of $(Na^+ + K^+)$-ATPase rather than a direct effect upon mitochondria. However, ouabain does not prevent respiratory stimulation by K^+ of brain slices incubated in conventional saline or Ringers medium [4,7—10] except in the absence of Ca^{2+} [4—6,8]. Therefore, the possible contribution of direct effects of K^+ and Na^+ upon mitochondria to the influence of these ions upon the metabolism of brain slices was assessed.

A new method for isolating mitochondria from rat brain was developed for the purpose of correlating monovalent cation permeabilities with the metabolic influences of K^+ and Na^+. Numerous isolation procedures have been described although two are most frequently employed [12,13]. The preparation developed by Ozawa et al. [12] yields brain mitochondria with good respiratory control but low maximal respiratory rates probably due to incomplete purification. A more highly purified preparation has been described by Clark and Nicklas [13] although its respiratory activity appears impaired, e.g. maximal succinate dehydrogenase activity does not increase in parallel with the cytochrome content of the mitochondrial fraction [13]. The isolation scheme employed in the present studies provides a balance between mitochondrial purity and structural and functional integrity. Stimulatory and inhibitory effects of K^+ and Na^+ upon respiration under different experimental conditions have been correlated with electrogenic and electrically silent fluxes of these ions.

Methods

Preparation of mitochondria. Male rats (100—150 g) are decapitated, the brain stem removed and the cortex and cerebellum immediately placed in ice-cold medium A (300 mM mannitol, 5 mM potassium glycylglycine and 0.1 mM potassium EDTA, pH 7.4). All manipulations are performed at 0—4°C. The brains are minced with scissors, rinsed with medium, and homogenized first with a loosely fitting Teflon pestle and glass homogenizer system (two strokes). This is repeated with a tightly fitting Teflon pestle (two strokes) with fresh medium A.

The homogenate is centrifuged at $1500 \times g$ for 8 min (Sorvall RC-2-B with a SS-34 rotor). The supernatant is centrifuged for 10 min at $10\,000 \times g$ to obtain the crude mitochondrial pellet. The upper white layer of the pellet is decanted with the supernatant. The pellet is resuspended in medium B ('salt medium') containing 200 mM mannitol, 8 mM potassium glycylglycine, 40 mM KCl, 4 mM NaCl, and 70 μM potassium EDTA, pH 7.4) by hand homogenization with a loosely fitting pestle and centrifuged at $1500 \times g$ for 2 min. The supernatant is centrifuged again for 2 min at $1500 \times g$ to remove heavy synaptosomes. The resulting supernatant (containing 'salt-washed' mitochondria) is centrifuged 10 min at $8000 \times g$ to sediment mitochondria. The pellet is

resuspended in medium A by hand homogenization and centrifugation for 10 min at 8000 × *g* repeated. Light layer mitochondria are judiciously decanted and the final mitochondrial pellet is resuspended with a pipette in the appropriate medium (usually 300 mM mannitol, 15 mM Tris/glycylglycine, 0.1 mM Tris/EDTA, pH 7.2).

Materials. Male Wistar rats were purchased from Hilltop Laboratories, Scottsdale, PA. Mannitol, rotenone, pyruvate, α-ketoglutarate, bovine serum albumin, ATP, NADH, and EGTA were obtained from Sigma Chemical Co., St. Louis, MO. Other chemicals were purchased from Fisher Scientific Co., Fair Lawn, NJ, except valinomycin (Calbiochem., Los Angeles, CA) and FCCP (a gift from Dr. P.G. Heytler, E.I. Dupont de Nemours and Co., Wilmington, DE).

Assay procedures. Oxygen uptake rates were measured polarographically with the Clark membrane electrode and swelling was monitored by absorbance changes at 600 nm with an Aminco DW-2 spectrophotometer.

Cytochrome contents were determined from room temperature difference spectra (extinction coefficients and wavelength pairs from Ref. 14). $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) was mesured at 37°C from the rate of inorganic phosphate liberation as determined by a modification of the method of Fiske and Subbarow [15]. The ouabain-sensitive $(Na^+ + K^+)$-ATPase activity of mitochondria pretreated with rotenone (0.2 μg/mg) was determined from the difference in the rate of ATP hydrolysis in the presence of 120 mM NaCl, 20 mM KCl, 40 mM Tris-HCl, 6 mM $MgCl_2$, and 5 mM Tris/ATP at pH 7.4 and 37°C with and without 0.3 mM ouabain.

Lactate dehydrogenase (EC 1.1.1.27) activity was determined at 25°C from the rate of NADH oxidation measured spectrophotometrically [16]. Cytochrome oxidase (EC 1.9.3.1) was assayed polarographically at 23°C essentially as described by Hamberger et al. [17] except mitochondria were solubilized with Triton X-100. Mitochondrial protein was determined by the method of Lowry et al. [18] using bovine serum albumin as standard.

Results

Rat brain mitochondria prepared by the 'salt wash' technique rapidly oxidized a variety of substrates (Table I). The maximal oxygen uptake rates obtained with these substrates were appreciably greater than reported for earlier preparations. Succinate, for example, supported the highest respiratory rate of 226 ngatoms O/min per mg protein compared to values of 150 and 80 for the preparations described by Clark and Nicklas [13] and Ozawa et al. [12], respectively. These mitochondria also retained functional integrity since respiratory control ratios with uncoupling agent (FCCP) ranged from 7 to 16 for the substrates tested (Table I). Control ratios with ADP were 10 or greater and P/O quotients with pyruvate (+ malate) as substrate were 2.7–2.9 (data not shown). In addition to these functional criteria, the criterion of mitochondrial purity was also satisfactorily met.

The purified mitochondria possessed a cytochrome oxidase specific activity of 3.3 μg atoms O/min per mg protein (see Methods) or eight times that of the original homogenate. The 'salt wash' procedure removed approximately 70% of

TABLE I

RESPIRATORY CONTROL AND MAXIMAL RESPIRATORY ACTIVITY OF RAT BRAIN MITOCHONDRIA

Rat brain mitochondria (approximately 1 mg protein/ml) were suspended in medium containing 300 mM mannitol, 10 mM Tris-HCl, 10 mM KCl, 5 mM potassium phosphate, 0.1 mM (potassium) EDTA, 1 mg bovine serum albumin/ml at 25°C and pH 7.2. The final concentration of substrates (Tris salts) was 5 mM except as indicated. Rotenone (0.2 μg/mg protein) was included with succinate. Maximal respiratory rates were determined with FCCP (0.1—0.4 μM). The values are the means (± S.E.) of four separate mitochondrial preparations.

Substrate	Maximal oxidation rate (ngatom O/min per mg)	Respiratory control index
Pyruvate, malate (1 mM)	203 ± 50	16.7 ± 3.2
α-Ketoglutarate	101 + 13	9.6 ± 1.9
Succinate (+ rotenone)	226 ± 42	7.1 ± 0.4
Glutamate, malate (1 mM)	95 ± 7	6.8 ± 0.9
β-Hydroxybutyrate	41 ± 6	3.4 ± 0.2
Endogenous	8 ± 4	

the plasma membrane marker, ouabain-sensitive ($Na^+ + K^+$)-ATPase, and the cytoplasmic marker, lactate dehydrogenase. The specific activity for lactate dehydrogenase (0.27 μmol NADH oxidized/min per mg was approximately 50% greater than that of the highly purified preparation of Clark and Nicklas [13]; however, the mitochondrial cytochrome content was about twice that of mitochondria obtained by the previously described method [13]. Values for cytochromes *b*, *c*, *a*, and a_3 were 0.27, 0.51, 0.30 and 0.29 nmol/mg protein, respectively (see Methods). These apparent differences in purity cannot be explained at the present time.

The ($Na^+ + K^+$)-ATPase activity (0.04 μmol P_i/min per mg) for these mitochondria from rat brain is comparable to that of bovine brain mitochondria purified by means of a discontinuous Ficoll/sucrose gradient [17]. Although Ozawa et al. [12] reported no measurable ($Na^+ + K^+$)-ATPase for their preparation, the assays were not performed at 37°C which is necessary to detect this activity in brain [19].

Further purification by the Ficoll gradient procedure [13] was not advantageous since respiratory control and maximal oxidative capacity were decreased nearly 50%.

To test the influences of K^+ and Na^+ upon respiration, choline chloride was used as a control and yielded respiratory rates on the average 20% greater than rates in mannitol medium. On the other hand, K^+ significantly stimulated basal respiration (Table II). The extent of stimulation depended upon the substrate and the particular mitochondrial preparation although K^+ caused partial uncoupling in all instances.

The maximal respiratory rate (+FCCP) was not altered appreciably by K^+ except for a significant but variable stimulation of pyruvate oxidation (Table II). This was not unexpected since K^+ is an activator of pyruvate dehydrogenase [20] which could limit electron transfer in the presence of excess uncoupling agent. It is noteworthy that K^+ did not increase maximal respiration rates in general but only with pyruvate in contrast to earlier reports that K^+ stimulates

TABLE II

INFLUENCE OF K^+ AND Na^+ UPON MITOCHONDRIAL RESPIRATION

Mitochondria (approximately 1 mg/ml) were suspended in media consisting of 125 mM KCl, NaCl or choline chloride plus 15 mM Tris/glycylglycine, 2 mM Tris/P_i, 0.2 mM Tris/EDTA and 1 mg albumin/ml at pH 7.2. Final substrate concentrations (Tris salts) were 5 mM except malate as indicated. Rotenone (0.2 μg/mg) was added with succinate. Maximal respiratory rates were determined with FCCP (0.1—0.4 μM), i.e. state 3u, and compared to the basal rates for six mitochondrial preparations. Data indicate the stimulation or inhibition of respiration and are expressed as percent greater or less than control with choline chloride.

Substrate	Potassium		Sodium	
	Basal rate	+FCCP	Basal rate	+FCCP
Succinate (+ rotenone)	+115 ± 32	−2 ± 7	+351 ± 24	−35 ± 3
α-Ketoglutarate	+56 ± 20	+5 ± 20	+385 ± 88	−14 ± 16
Glutamate, malate (1 mM)	+65 ± 37	+8 ± 10	+363 ± 82	−17 ± 19
Pyruvate, malate (1 mM)	+79 ± 27	+26 ± 23	+742 ± 151	−7 ± 21

respiration with various substrates [21,22]; see Discussion.

Whereas K^+ caused modest uncoupling, Na^+ almost completely uncoupled respiration (Table II). Basal oxygen uptake rates were about four times as rapid in Na^+ medium compared to choline. The degree of uncoupling by Na^+ was substrate dependent as in the case of K^+. It should be stressed that no effects of Na^+ or K^+ upon respiration were sensitive to ouabain (tested at 300—500 μM; data not shown). Thus the influence of these ions could not be attributed to contaminating (Na^+ + K^+)-ATPase.

As shown in Table II, Na^+ inhibited uncoupler-stimulated respiration rates. Inhibition of succinate oxidation was more than twice that of other substrates tested. Only slight and variable inhibition of α-ketoglutarate or glutamate oxidation was observed and Na^+ had little apparent effect upon pyruvate oxidation. Inhibition of pyruvate oxidation may have been underestimated due to simultaneous activation of the dehydrogenase which can occur with Na^+ under certain conditions [20]. Sodium could inhibit substrate oxidation by exchanging with protons (a Na^+/H^+ antiport) thereby reducing the pH gradient necessary for substrate uptake [23—25].

Uncoupling by Na^+ and K^+ required EDTA and was markedly decreased by Mg^{2+} (Table III). It is noteworthy that uncoupling by K^+ was greater than Na^+ in the presence of Mg^{2+}, i.e. under possible in situ conditions. In addition to EDTA, P_i was necessary for maximal uncoupling by either Na^+ or K^+. For example, if P_i was omitted, uncoupling of succinate oxidation by Na^+ decreased from over 300% (Table II) to approximately 150%. Furthermore, addition of *N*-ethylmaleimide (80 nmol/mg) to prevent reaccumulation of endogenously released P_i [26] decreased Na^+-dependent uncoupling to only 70%, i.e. about one-fourth, and completely blocked enhancement by added P_i. Exogenous and presumably endogenous P_i probably promoted uncoupling by increasing cation conductance (see Discussion) and could not be replaced by acetate. Phosphate has been reported to enhance the monovalent cation permeability of rat liver [27] and heart [28] mitochondria.

Inhibition of maximal or uncoupler-stimulated respiration by Na^+ was much

TABLE III

EDTA REQUIREMENT FOR UNCOUPLING BY K^+ OR Na^+

Mitochondria were incubated in medium in the presence of 0.1 mM Tris/EDTA for 1 min prior to addition of $MgCl_2$ to obtain reproducible maximal rates of succinate oxidation. The experimental conditions were otherwise identical to those described for Table II. The values are averages for three preparations. The respiratory rate data are expressed as percent greater or less then isotonic choline chloride control.

Salt	Respiratory rate	
	0.1 mM EDTA	0.1 mM $MgCl_2$
NaCl		
Basal	+274	+11
+FCCP	−39	−23
KCl		
Basal	+91	+65
+FCCP	−5	−4

less sensitive to Mg^{2+} than uncoupling (Table III). Raising the level of EDTA to 1 mM or Mg^{2+} to 5 mM did not alter the pattern of results. To shed light on the mechanisms involved in Na^+ and K^+ influences upon respiration, mitochondria were tested for cation permeabilities by measuring passive swelling in the appropriate salts. Representative tracings are shown in Fig. 1.

Swelling was most rapid in sodium acetate indicating brain mitochondria possessed an active Na^+/H^+ antiport and a less active K^+/H^+ antiport according to swelling in potassium acetate. Swelling also occurred in Cl^- salts: $Na^+ > K^+ > NH_4^+$ *. Swelling in NH_4Cl (or KCl and NaCl) could proceed by either of two mechanisms: cation conductance plus Cl^- conductance or by a combination of electrically neutral exchanges. In the latter case, NH_3 permeation and protonation by H_2O to form NH_4^+ (equivalent to neutral NH_4^+/H^+) or neutral Na^+ or K^+/H^+ exchange together with neutral Cl^-/OH^- exchange could cause swelling. The rate of swelling was therefore measured as a function of cation concentration as one means of distinguishing between conductances (e.g. NH_4^+ or K^+ with Cl^-) and neutral exchange processes. If NH_3 were the only permeant species and since it permeates membranes so rapidly, the rate of swelling should be largely independent of NH_4^+ concentration. This was found to be the case as shown in Fig. 2.

The rate of swelling in NH_4^+ was the same at 1.25 mM as 125 mM. In contrast, the rate of K^+-dependent swelling (attributed to K^+ conductance) increased nearly two-fold over this same concentration range. Thus NH_3 rather than NH_4^+ was the principal permeating species and swelling in NH_4Cl provided a measure of maximum Cl^-/OH^- exchange activity. Swelling in NaCl and KCl could therefore be corrected for possible contributions of neutral exchanges, i.e. Na^+ or K^+/H^+ plus Cl^-/OH^-, to more reliably assess Na^+ and K^+ conductances under the desired experimental conditions. Henceforth, all values cited for cation conductances have been corrected accordingly. However, these are

* These measurements were evidently not complicated by swelling of contaminating synaptosomes. Synaptosomes obtained from the low-speed spin following mannitol-salt washing (see Methods) exhibited much less swelling in both Cl^- ($K^+ > Na^+$) and acetate salts ($Na^+ > K^+$).

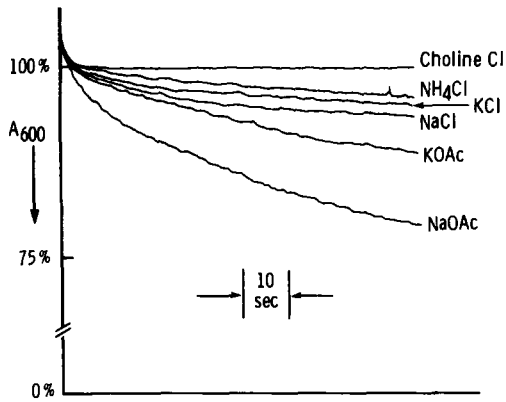

Fig. 1. Passive mitochondrial swelling in isotonic salts. Mitochondria (0.15 mg/ml) were added to a cuvette equipped with rapid magnetic stirring and the decrease in absorbance at 600 nm (indicating mitochondrial swelling) was recorded continuously. The medium contained 125 mM salt plus 15 mM Tris/glycylglycine and 0.2 mM Tris/EDTA at pH 7.2. Mitochondria were pretreated with rotenone (0.2 μg/mg protein.

apparent values only. Although swelling measurements are widely employed for identifying permeability types [29,30], they are indirect measures of ion fluxes.

The effects of EDTA and Mg^{2+} upon cation permeabilities are summarized in Table IV. The K^+ conductance was decreased by Mg^{2+} to about 50% its value in EDTA. Valinomycin accelerated swelling in KCl six-fold implying that Cl^- conductance was not limiting. The Na^+ conductance was exceedingly sensitive to Mg^{2+} which lowered its value to only 10% of that in EDTA. The Na^+/H^+

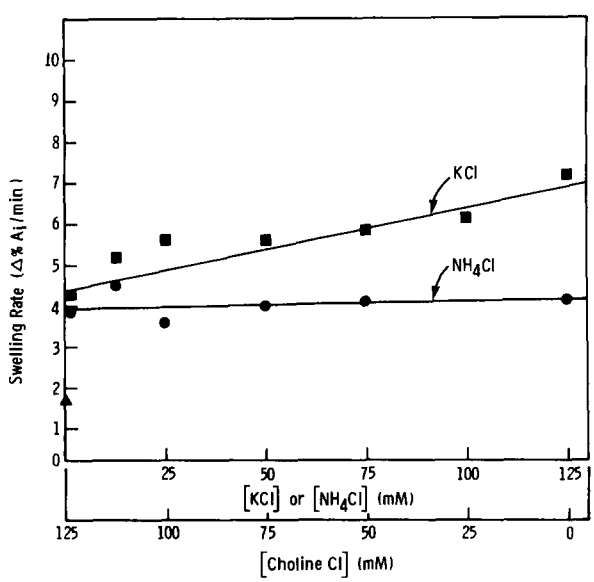

Fig. 2. Relative contributions of chloride exchange and chloride conductance to mitochondrial swelling. Experimental conditions were identical to those described in the legend to Fig. 1 except 1 mM $MgCl_2$ was added with NH_4Cl and 0.2 mM Tris/EDTA with KCl.

TABLE IV

CATION CONDUCTANCE AND CATION/H^+ EXCHANGE OF RAT BRAIN MITOCHONDRIA

Passive swelling was measured as shown in Fig. 1. The rate of swelling is expressed as the change/min in the percent of initial absorbance at 600 nm ($\Delta\%\ A_i$/min). Swelling rates were calculated from the slope of absorbancy decrease 8 s after addition of mitochondria. Valinomycin (20 ng/mg protein) was added as indicated.

Salt	Swelling rate ($\Delta\%\ A_i$/min)	
	0.2 mM EDTA	1 mM $MgCl_2$
KCl	2.0	1.1
KCl + valinomycin	N.D.	6.1
NaCl	4.7	0.5
Potassium acetate	11.1	11.6
Sodium acetate	28.9	26.8

antiport was 2—3 times as active as the K^+/H^+ antiport and neither was affected by EDTA or Mg^{2+}. The inhibition of maximum respiration by Na^+, attributed to Na^+/K^+ exchange (Table II), was not appreciably diminished by Mg^{2+} (Table III). This is in line with the greater Na^+/H^+ than K^+/H^+ antiport activity and its insensitivity to Mg^{2+} (Table IV).

It should be noted that cation conductances were substantially lower than neutral exchange values according to passive swelling measurements (Table IV). Although these comparisons established the chelator and Mg^{2+} sensitivities of these permeabilities, cation conductances were probably underestimated. Swelling was proportionately more rapid in NO_3^- or SCN^- than Cl^- salts suggesting anion conductance was limiting (data not shown). These factors were presumably circumvented under energized conditions where rapid H^+ ejection and electrical potential generation could provide adequate charge compensation for inward cation conductance (see Table V).

Cation conductance evidently was the major factor in uncoupling. Monovalent cation conductance was maximal in the presence of EDTA which correlated with the EDTA requirement for uncoupling by Na^+ and K^+ (Table III). Furthermore, the two-fold higher conductance value for K^+ compared to Na^+ in the presence of Mg^{2+} (Table IV) correlated with the greater uncoupling by K^+ than Na^+ when Mg^{2+} was present (Table III). The insensitivity of cation/H^+ antiport activity to EDTA and Mg^{2+} also indicated cation conductance rather than exchange dominated in uncoupling. This conclusion was further tested by titrating mitochondria with the K^+-conducting ionophore, valinomycin [32], to establish a quantitative relationship between uncoupling and K^+ conductance as deduced from swelling rates. This could then be used to calculate expected values for respiratory stimulation from experimentally determined swelling rates with Na^+ and K^+.

Conditions were determined for obtaining maximal swelling rates as the best approximations of cation conductance under metabolizing conditions. The inward conductance of cations could be diminished significantly by the backflow of K^+ or Na^+ via neutral exchange with protons extruded during electron transfer. The conditions sustaining maximal swelling rates for valinomycin plus K^+ and K^+ or Na^+ alone are provided in Table V. In each instance, P_i plus ATP

TABLE V

CONDITIONS FOR MAXIMAL RATES OF MITOCHONDRIAL SWELLING

Mitochondria were equilibrated with media containing 125 mM KCl, 15 mM Tris/glycylglycine, 0.2 mM Tris/EDTA at pH 7.2 before addition of 5 mM Tris/succinate. Swelling was initiated by addition of 2 mM Tris/P_i followed 5 s later by valinomycin (15 ng/mg protein) or by valinomycin alone. Where indicated, 0.2 mM Tris/ATP was included in the equilibration medium and EDTA was replaced with 0.2 mM Tris/EGTA plus 1 mM $MgCl_2$. In the case of NaCl, swelling was initiated by simultaneous addition of all components including succinate. The results are the average of two experiments and are not corrected for Cl^-/OH^- exchange. The conditions are otherwise as described in the legend of Fig. 1.

Addition(s)	Swelling rate (Δ% A_i/min)		
	KCl + valinomycin	KCl	NaCl
EDTA	22	6	7
EGTA, Mg^{2+}	21	5	4
EDTA, P_i	59	16	36
EDTA, ATP	50	3	16
EDTA, ATP, P_i	75	22	54
EGTA, Mg^{2+}, ATP, P_i	68	16	9

stimulated the rate of swelling maximally.

Valinomycin-dependent swelling was accelerated more than two-fold by P_i or ATP and stimulation by P_i plus ATP was somewhat less than additive. Approximately the same rates were obtained with EDTA or Mg^{2+} plus EGTA; the latter combination was employed with valinomycin (cf. Table VI) in preference to EDTA to eliminate interferences due to spontaneous swelling in KCl.

The swelling rates in K^+ or Na^+ without ionophore were also stimulated several fold by P_i (Table V). Although ATP stimulated Na^+-dependent swelling, it slightly inhibited swelling in K^+. This difference cannot be explained at the present time. Nevertheless, the combination of ATP and P_i yielded maximal swelling rates with K^+ and Na^+ as observed with valinomycin. Thus optimum conditions for swelling and therefore estimation of cation conductances were quite similar in all three cases permitting side by side comparisons between valinomycin, the model K^+ conductor, and natural K^+ and Na^+ conductances. The only obvious difference was the EDTA enhancement and Mg^{2+} inhibition of swelling in K^+ and Na^+ (Tables IV and V) in contrast to swelling stimulated by valinomycin.

Linear increases in the rates of mitochondrial swelling in KCl (Fig. 3A) and basal respiration (Fig. 3B) were obtained with valinomycin additions up to 20 ng/mg protein. P_i plus ATP (with or without oligomycin) had no effect upon uncoupling although they stimulated the rate of swelling (Table V) unlike the P_i enhancement of Na^+ and K^+-dependent uncoupling described earlier and attributed to an increase in cation conductance. Quantitative correlations between natural Na^+ and K^+ conductances and uncoupling by these ions are provided in Table VI. Observed values for uncoupling are compared to those predicted according to the relationship between K^+ conductance or swelling and respiratory stimulation established with valinomycin (Fig. 3A and B).

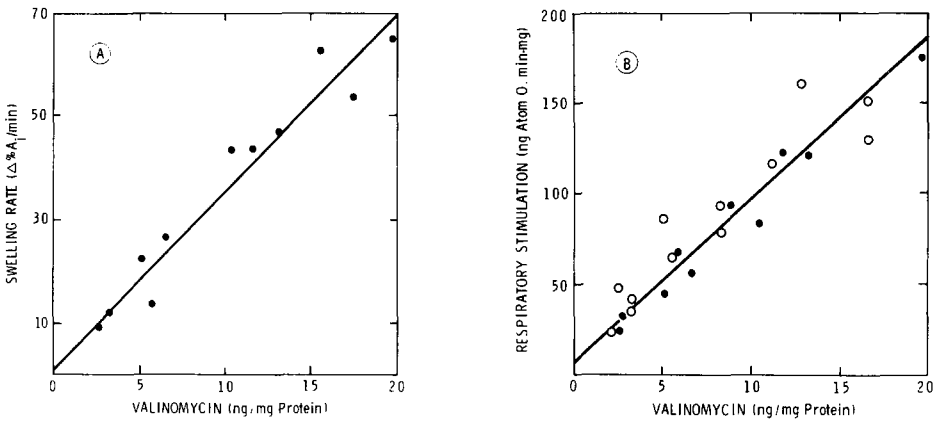

Fig. 3. (A) Valinomycin-dependent swelling of mitochondria. Mitochondria (pretreated with rotenone) were equilibrated with media containing 125 mM KCl, 15 mM Tris/glycylglycine, 0.2 mM Tris/EGTA, 1 mM $MgCl_2$ at pH 7.2 before adding 5 mM Tris/succinate. Swelling was initiated by addition of 2 mM Tris/P_i followed 5 s later by valinomycin (n = 3). (B) Stimulation of respiration by valinomycin. Valinomycin titrations were performed on mitochondria (0.5 mg/ml) suspended in media containing 125 mM KCl, 15 mM Tris/glycylglycine, 5 mM Tris/succinate, rotenone (0.2 μg/mg protein), and 1 mM $MgCl_2$ at pH 7.2 (○, n = 4) or the same medium supplemented with 2 mM Tris/P_i, 0.2 mM Tris/ATP, 0.2 mM Tris/EGTA, and 2 μg oligomycin (●, n = 3).

The results of Table VI demonstrate an exceptionally good agreement between calculated and observed values for stimulation of respiration by Na^+ and K^+ whether conductances are high with EDTA present or relatively low when Mg^{2+} is added.

After ascertaining certain of the requirements for monovalent cation permeabilities and their influence upon oxidative metabolism these were considered in light of conditions presumed to prevail in situ. The concentration dependences were determined for Na^+ and K^+ individually to assess their effects independently at physiological levels. The titrations of Fig. 4 are expressed in terms of respiratory control ratios since they encompass effects upon both basal and maximal respiration.

TABLE VI

CORRELATION BETWEEN SWELLING AND RESPIRATORY STIMULATION

Swelling rates were employed to calculate stimulation of respiration by Na^+ and K^+ from the valinomycin titrations of Fig. 3A and B. These rates (n = 3) were measured in NaCl or KCl as described in the legend to Fig. 3A except swelling was initiated by P_i alone. When EDTA was added in place of Mg^{2+} plus EGTA, swelling was initiated by simultaneous addition of EDTA, P_i, ATP and succinate. Observed values for respiratory stimulation were determined in a separate series of experiments. Oxygen uptake rates were determined essentially as described in Table II with succinate (5 mM) as substrate and choline chloride controls. Either 1 mM $MgCl_2$ (n = 4) or 0.2 mM Tris/EDTA (n = 9) was added as indicated.

Experimental conditions	Stimulation of respiration (ngatoms O/min per mg)	
	Calculated	Observed
NaCl (EDTA)	148	142 ± 29
KCl (EDTA)	53	46 ± 9
KCl (Mg^{2+})	31	29 ± 4
NaCl (Mg^{2+})	15	8 ± 7

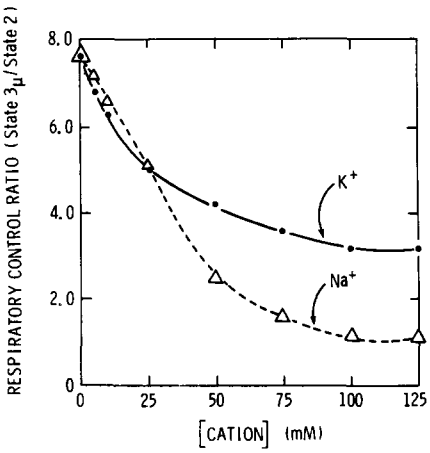

Fig. 4. Uncoupling of respiration by Na^+ and K^+. Mitochondria (0.6 mg/ml) were suspended in media consisting of the indicated concentrations of NaCl or KCl plus sufficient choline chloride to maintain constant tonicity. Other media inclusions were 15 mM Tris/glycylglycine, 5 mM Tris/succinate, 2 mM Tris/P_i, 0.2 mM Tris/EDTA, 1 mg albumin/ml and 0.2 μg rotenone/mg protein at pH 7.2.

Respiratory control decreased monotonically as the concentration of K^+ or Na^+ was increased (Fig. 4). Half-maximal effects were obtained with approximately 25 mM K^+ or Na^+. Between 20 and 30 mM Na^+ (its approximate cytosolic concentration) respiratory control was reduced by 25—45% whereas a physiological level of K^+ (125 mM) decreased control about 60%.

More pertinent to the question of in situ influences of K^+ and Na^+ were their combined effects in the presence of Mg^{2+} since this ion inhibited uncoupling by either cation, particularly Na^+ (cf. Table III). These results are summarized in Table VII. At 1 mM Mg^{2+}, i.e. roughly the free intracellular concentration [33], uncoupling by K^+ was moderately reduced and that by Na^+ markedly so. Magnesium similarly influenced uncoupling by the combinations of K^+ and Na^+ tested. Thus the net effect of Mg^{2+} was to render K^+ a more significant regula-

TABLE VII

THE Mg^{2+} DEPENDENCE OF MONOVALENT CATION INFLUENCES UPON RESPIRATORY CONTROL

Mitochondria (0.5 mg/ml) were suspended in media consisting of the indicated concentrations of KCl and NaCl except in the control (first) experiment in which choline chloride (125 mM) was substituted. In addition, media contained 15 mM Tris/glycylglycine, 5 mM Tris/succinate, 5 mM Tris/P_i, 1 mg albumin/ml and 0.2 μg rotenone/mg protein at pH 7.2. Either 1 mM $MgCl_2$ or 0.2 mM EDTA was added as indicated.

Salt (mM)		Respiratory control index	
KCl	NaCl	$-Mg^{2+}$	$+Mg^{2+}$
0	0	6.7	6.4
125	0	2.7	4.0
100	25	1.9	3.5
25	100	1.0	4.0
0	125	1.0	5.0

tor of respiration than Na^+ and to 'buffer' respiratory control against changes arising from alterations in the K^+/Na^+ ratio.

Discussion

The influences of Na^+ and K^+ upon the respiration of brain mitochondria have been related to their permeabilities. Uncoupling correlates well with cation conductance whereas inhibition of maximal respiratory rates is most readily explained in terms of neutral cation/H^+ exchange.

The correlation between cation conductance and uncoupling was substantiated by a linear relationship between uncoupling by valinomycin and the rate of swelling (i.e. K^+ conductance) and the close agreement between observed and predicted extents of uncoupling by Na^+ and K^+ calculated from their conductance values.

Since uncoupling is generally attributed to diminution of the electrochemical gradient of protons ($\Delta\mu_{H^+}$) [31] and the conductance of cations only affects the membrane potential ($\Delta\psi$), sustained uncoupling occurs only if ΔpH is simultaneously suppressed. This could occur by any of three mechanisms: neutral anion/OH^- or cation/H^+ exchange or increased H^+ conductance, e.g. due to swelling [31,34—36] and nonspecific changes in permeability [31,37].

Several observations argue against swelling as the cause of uncoupling described here. Mitochondria swollen in hypotonic media to the same extent achieved in swelling experiments were not significantly uncoupled. In valinomycin titrations (Fig. 3A and B), uncoupling was proportional to the rate and not the extent of swelling which was nearly constant. Finally, P_i increased net swelling stimulated by valinomycin but not uncoupling (Fig. 3B).

A depression of ΔpH by anionic metabolite uptake could support uncoupling although succinate (employed in most experiments) is accumulated by succinate/P_i (or succinate/malate) exchange [38] which would not collapse ΔpH. Phosphate should collapse ΔpH, however, it did not enhance uncoupling by valinomycin. However, it is possible the pH gradient was already attenuated by endogenous P_i or other transportable anions.

An alternative or additional mechanism for suppressing ΔpH to explain uncoupling is neutral cation/H^+ exchange. Brain mitochondria clearly possess K^+/H^+ and Na^+/H^+ exchange according to measurements of passive swelling in acetate salts (Fig. 1 and Table IV) although these rates were lower than maximum apparent cation conductances (Table VII). Influx via cation/H^+ exchange appears, however, to be regulated in a complex fashion and not simply related to efflux [39] which could differ considerably according to the pH gradient under respiring conditions. Until such time as K^+ and Na^+ 'turnover' rates can be measured and directly related to conductance and neutral exchange, this model of uncoupling by cation cycling via conductance plus exchange (see Refs. 40 and 41) must remain a working hypothesis.

The molecular basis of monovalent cation conductance is unknown. A key regulatory role for Mg^{2+} is indicated by enhancement of conductance by EDTA (and not EGTA) and its attenuation by exogenous Mg^{2+}. This is in keeping with the results of Brierley and coworkers that removal of membrane-bound Mg^{2+} from heart mitochondria exposes 'pores' that conduct monovalent cations

[42]. Phosphate appears to increase cation conductance since it enhances uncoupling by Na^+ and K^+ which is limited by their conductances. However, since anion conductance evidently limited passive swelling rates, this could not be demonstrated more directly. Phosphate may act indirectly by altering endogenous intramitochondrial Mg^{2+} since treating brain mitochondria with EDTA plus the ionophore A23187, which releases endogenous Mg^{2+} [42,43], produces Na^+-dependent uncoupling in the absence of P_i [44]. On the other hand, the Ca^{2+} 'permease' inhibitor, ruthenium red [45], significantly inhibits Na^+ conductance and Na^+-dependent uncoupling [44]. Thus monovalent cation conductance and its regulation by Mg^{2+} may involved the Ca^{2+}-binding protein [46] as proposed by Krall [47] or other glycoproteins with an affinity for ruthenium red whose membrane binding is similarly influenced by Mg^{2+}, etc.

In previous studies with brain mitochondria [13,21,22], exogenous K^+ was required for maximal oxidation rates with various substrates, whereas K^+ stimulated only pyruvate oxidation in the present studies. The K^+ stimulation reported previously may have reflected potassium depletion since the maximal respiratory activity of brain mitochondria correlates with K^+ content [48]. The potassium levels of 'salt-washed' mitochondria employed in the present studies were approximately the same (230 nequiv./mg) as the preparation of Ozawa et al. [48] although they possessed about 40% more 'free K^+' (released with valinomycin plus uncoupler) and roughly twice the 'free' and 'bound K^+' (released only by Triton X-100) of the Clark and Nicklas preparation [13]. Perhaps inclusion of K^+ in the isolation medium (see Methods) is responsible for these differences.

Although extrapolation from in vitro results is admittedly tenuous, one objective of the present study was to assess whether monovalent cations can directly influence respiration in vivo, i.e. not simply by activating ($Na^+ + K^+$)-ATPase. Only results with physiological levels of Mg^{2+} are considered since this is presumed to apply in vivo.

In the presence of Mg^{2+}, K^+ stimulated basal respiration about 50% and Na^+ had little effect in line with observations that the substantial stimulation of brain slice respiration by K^+ occurs by activating ($Na^+ + K^+$)-ATPase [4–6,8]. On the other hand, the basal respiration of brain slices treated with oligomycin is stimulated about 40% by K^+ [4] in agreement with the present in vitro studies. Previous in vitro studies would predict as much as a doubling of maximal respiration, e.g. linked to ATP synthesis [13,21,22,49], yet this was not observed with brain homogenates [6]. In the present studies, K^+ did not stimulate maximal respiration with either uncoupling agent (Table II) or ADP (data not presented) although respiration with pyruvate was accelerated somewhat (25%) by K^+. This is consistent with the view that this ion is involved in rapid aerobic glycolysis by brain [1–3], perhaps by activating pyruvate dehydrogenase [22]. Finally, Na^+ was shown to inhibit respiration most likely via Na^+/H^+ exchange. Sodium inhibition of the 'ouabain-insensitive respiration' of rat brain slices (and homogenates) has been correlated with accumulation of intracellular Na^+ [6] which is compatible with operation of Na^+/H^+ exchange in situ. Thus results obtained with mitochondria isolated by the 'salt wash' technique resemble the in situ behavior of mitochondria in several aspects.

Despite these general similarities, the monovalent cation permeability of

brain mitochondria in situ is not yet known and must be established to properly assess results of these and other in vitro studies.

Acknowledgements

This investigation was supported by funds from Public Health Service Grant No. CA 11766 from the National Cancer Institute; P.A.B. is the recipient of a N.I.H. Predoctoral Fellowship (GM 00446).

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