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REGULATION OF THE MONOVALENT CATION PERMEABILITY OF BRAIN MITOCHONDRIA

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The Na^+ and K^+ conductances of rat brain mitochondria were estimated from rates of metabolically dependent swelling and uncoupling of respiration. These were maximal in the presence of EDTA plus P_i. P_i could not be replaced with acetate. Na^+ conductance was greater than that of K^+ and was therefore examined in greater detail. According to the influences of *N*-ethylmaleimide, internal P_i (exogenous and perhaps endogenous) promoted Na^+ permeability. Treatment with the ionophore A23187 obviated the P_i requirement although EDTA was still necessary. The stimulation by EDTA with P_i or A23187 and inhibition by exogenous Mg^{2+} suggested endogenous polyvalent cations could also regulate Na^+ conductance. The influence of these substances upon endogenous Mg^{2+} (and Ca^{2+}) levels is consistent with such a role of membrane-bound Mg^{2+}. Low levels of ruthenium red (150 pmol/mg) inhibit Na^+ permeation, indicating that the number of 'sites' or 'channels' involved may be small. The Ca^{2+} uniport is not directly involved in Na^+ flow according to its greater sensitivity to inhibition by ruthenium red.

Introduction

Na^+- and K^+-dependent uncoupling of rat brain mitochondria can be explained quantitatively in terms of limiting conductances to these ions [1]. The influences of Na^+ and K^+ upon the respiration of isolated brain mitochondria agree well with their effects upon mitochondrial respiration in situ [2–7]. Hence, the mitochondrial permeabilities in vitro and in situ appear to be quite similar. Isolated brain mitochondria are therefore particularly useful in studies of factors regulating the flow of monovalent cations.

The present studies deal principally with the apparent Na^+ conductance of mitochondria from brain [1,8] compared to other tissues (see Refs. 9 and 10 for reviews). The results indicate that Mg^{2+} (presumably membrane bound) is particularly important in regulation of monovalent cation permeability as in mitochondria from other tissues. A limited number of sites or channels (nominally one per cytochrome) is involved in Na^+ conductance based upon titrations with the Ca^{2+} 'permease' inhibitor, ruthenium red [11,12]. Although ruthenium red inhibits, the Ca^{2+} permease [13] is not directly involved in Na^+ conductance.

Experimental Procedure

Materials

Male Wistar rats were purchased from Hilltop Laboratories, Scottsdale, PA. Mannitol, rotenone, bovine serum albumin, ATP, EDTA, EGTA and ruthenium red were obtained from Sigma Chemical Co., St. Louis, MO. Valinomycin was purchased from Calbiochem, Los Angeles, CA, FCCP was a gift from

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Trien, triethylenetetramine.

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Dr. P.G. Heytler, E.I. Du Pont de Nemours and Co., Wilmington, DE and A23187 was kindly provided by Dr. R.J. Hosley, Eli Lilly and Co., Indianapolis, IN. All other chemicals were obtained from Fisher Scientific Co., Fair Lawn, NJ.

Mitochondria were isolated from the cortices and cerebella of male rats (100–150 g) by a modification of the method of Ozawa et al. [14] as described in detail elsewhere [1].

Assay procedures

Oxygen uptake rates were measured polarographically with a Clark membrane electrode and swelling was determined by absorbance changes recorded continuously at 600 nm with an Aminco DW-2 spectrophotometer. Divalent cations were determined on 10% HNO_3 extracts with a Perkin Elmer atomic absorption spectrophotometer (Model 303). Mitochondrial protein was assayed by the method of Lowry et al. [15] using bovine serum albumin as standard.

Results

General features of the K^+- and Na^+-dependent swelling and uncoupling of brain mitochondria, attributed to uptake by inward conductance of these ions [1], are provided in Fig. 1. Metabolically dependent swelling is equated with electrophoretic cation translocation or conductance as occurs with K^+ in the presence of valinomycin (Fig. 1), i.e., driven by the transmembrane electrical potential [16]. In the absence of ionophore, swelling is more rapid in Na^+ than K^+ and uncoupling is greater, i.e., basal respiration is increased 4-fold by Na^+ and 2-fold by K^+ compared to the control with choline chloride. Therefore, the Na^+ conductance of brain mitochondria is greater than that of K^+ (Fig. 1). Both require EDTA and are inhibited by exogenous Mg^{2+} [1,8,17]. Phosphate is also required, as shown in Fig. 2.

Na^+- or K^+-dependent swelling occurred with phosphate but not acetate. In contrast, the rate of swelling with valinomycin ($+K^+$) was the same with phosphate as with acetate. Evidently, phosphate does not stimulate Na^+- and K^+-dependent swelling by simply collapsing the pH gradient (via P_i/OH^- exchange) and indirectly increasing the membrane potential [18].

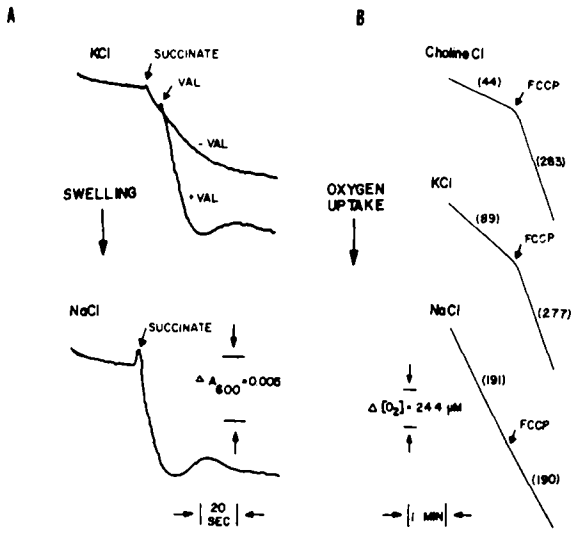

Fig. 1. (A) Cation conductance and swelling. (B) Cation-dependent uncoupling. Mitochondria were added to a cuvette equipped with rapid magnetic stirring and the decrease in absorbance (indicating mitochondrial swelling) was recorded continuously. The suspending medium contained 125 mM KCl or NaCl, 15 mM Tris-glycylglycine, 0.2 mM Tris-EDTA, 2 mM Tris-phosphate and 0.2 mM Tris-ATP at pH 7.2 plus rotenone-treated (0.2 μg/mg protein) mitochondria (0.15 mg/ml). Swelling was initiated by addition of Tris-succinate (5 mM) followed by valinomycin (VAL) (15 ng/mg protein), when included. Respiration was measured with mitochondria (0.5 mg/ml) suspended in medium containing 125 mM levels of the indicated salts. The conditions were otherwise the same as for swelling measurements except 0.1% bovine serum albumin was included and Tris-STP was omitted. FCCP (0.2 μM) was added as indicated to obtain maximal uncoupling.

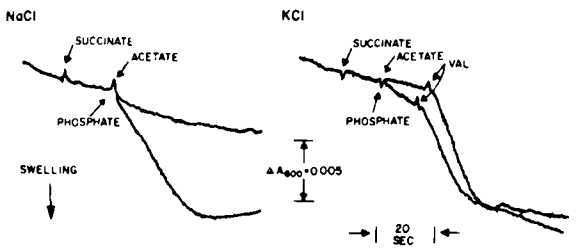

Fig. 2. The conditions were essentially identical to those described in the legend to Fig. 1 except Tris-acetate (2 mM) was added in place of phosphate as indicated, ATP was omitted and swelling was initiated with phosphate followed by valinomycin (VAL) (when included).

Uncoupling by Na^+ was also increased by exogenous phosphate (Table I) and comparable results were obtained with K^+ (data not shown). Henceforth, only results with Na^+ are presented. *N*-ethylmaleimide was employed to test for a possible influence of endogenous phosphate, by blocking the uptake of P_i released anaerobically [19,20]. Endogenous P_i is evidently involved, since uncoupling by Na^+ was inhibited about 50% by *N*-ethylmaleimide. Enhancement of uncoupling by exogenous phosphate was nearly completely blocked by *N*-ethylmaleimide. Acetate did not stimulate uncoupling by Na^+ in harmony with the conclusion that phosphate facilitates swelling (Fig. 2) by effecting permeability.

Brain mitochondria were depleted of divalent cations by treating with the ionophore A23187 [21, 22] as in studies with heart mitochondria [23] to determine whether or not this influenced Na^+ effects on respiration.

As shown in Table II, treatment with A23187 supported Na^+-dependent uncoupling providing EDTA was also present. EGTA could not replace EDTA. A23187 increased basal respiration to an extent comparable to that with P_i, i.e., treatment with the ionophore obviated the P_i requirement for uncoupling by Na^+. Controls with choline indicated that the uncoupling with A23187 was strictly Na^+ dependent. Since A23187 causes release of endogenous Mg^{2+} and Ca^{2+} [21–24], the levels of these ions were determined under control conditions and those sustaining Na^+-dependent uncoupling.

TABLE I

PHOSPHATE REQUIREMENT FOR Na^+-DEPENDENT UNCOUPLING

The conditions were the same as for the respiration experiments of Fig. 1. Tris-phosphate (2 mM), *N*-ethylmaleimide (60 nmol/mg) and Tris-acetate (2 mM) were added as indicated. The values are the means (±S.D.) for three preparations.

Cation	Addition(s)	Respiratory rate (ngatom O/min per mg)
$Choline^+$	P_i	40 ± 5
Na^+	none	98 ± 10
Na^+	P_i	166 ± 24
Na^+	*N*-ethylmaleimide	68 ± 10
Na^+	P_i, *N*-ethylmaleimide	74 ± 2
Na^+	Acetate	110 ± 31

TABLE II

INFLUENCE OF THE IONOPHORE A23187 UPON UNCOUPLING BY Na^+

Mitochondria (0.7 mg/ml) pretreated with 0.2 μg rotenone/mg were suspended in medium containing 125 mM NaCl or choline chloride, 15 mM Tris-glycylglycine, 0.1% bovine serum albumin at pH 7.2 plus, as indicated, Tris-EDTA or Tris-EGTA (0.2 mM), Tris-P_i (2 mM) and A23187 (0.5 μg/mg); the values are means (±S.D.) for at least three preparations. n.d., not determined.

Cation	Addition(s)	Respiratory rate (ngatom O/min per mg)	
		−A23187	+A23187
Na^+	EDTA	69 ± 18	138 ± 3
Na^+	EGTA	49 ± 1	54 ± 4
Na^+	EDTA, P_i	155 ± 12	n.d.
$Choline^+$	EDTA	37 ± 2	47 ± 4
$Choline^+$	EDTA, P_i	36 ± 1	n.d.

Brain mitochondria retain typical quantities of Mg^{2+} (37 nmol/mg) plus a rather high endogenous Ca^{2+} level (33 nmol/mg). Brief incubation in medium containing oxidizable substrate lowered both Mg^{2+} and Ca^{2+} (to 26 and 30 nmol/mg, respectively). The simplest comparison between uncoupling and control conditions is: EDTA + A23187, which sustains near maximum uncoupling and EGTA + ionophore, which does not uncouple (Table II). The Mg^{2+} content of mitochondria treated with EDTA was lower by 2 nmol/mg (11.0 ± 2.0 nmol Mg^{2+}/mg) compared to EGTA treatment (13 ± 3 nmol/mg protein; Table III). There was no statistically significant difference in the Ca^{2+} content of mitochondria treated with EDTA or EGTA (4 ± 3 and 6 ± 3 nmol Ca^{2+}/mg, respectively). These results are quite similar to those obtained with beef heart mitochondria in which a small fraction of Mg^{2+} (approx. 1 nmol/mg) regulates monovalent cation permeability and swelling [23]. However, there is a high 'background' of bound Mg^{2+} (and Ca^{2+}) in brain mitochondria despite treatment with A23187. These levels (11–13 nmol Mg^{2+}/mg and 4–5 nmol Ca^{2+}/mg; Table III) are significantly greater than in mitochondria from heart which retain only 2–4 nmol Mg^{2+}/mg after A23187 treatment [23]. Therefore, establishing the minimum level and particular pool of Mg^{2+} controlling mem-

TABLE III

Mg^{2+} AND Ca^{2+} CONTENTS OF MITOCHONDRIA SUBJECTED TO DIFFERENT TREATMENTS

The cation contents of unincubated mitochondria were: Mg^{2+}, 37 ± 10 and Ca^{2+}, 33 ± 7 nmol/mg. Mitochondria pretreated with rotenone (0.2 μg/mg) were incubated (at final average concentrations of 1.1 mg/ml) in media containing 125 mM NaCl, 15 mM Tris-glycylglycine, 5 mM Tris-succinate, 0.1% bovine serum albumin at pH 7.2 for 2 min. Where indicated, Tris-EDTA (0.2 mM), Tris-P_i (2 mM), Tris-EGTA (0.2 mM), or A23187 (0.5 μg/ml) were added initially. The suspension was centrifuged at 15 000 × *g* for 10 min and the pellet rinsed three times before extracting Mg^{2+} and Ca^{2+} with 10% HNO_3 plus 2000 ppm $LaCl_3$. The cation contents were determined by atomic absorption spectroscopy. The values are means (±S.D.) for at least five mitochondria preparations and statistical significances between divalent cation values (compared to the appropriate controls) were determined from analysis of the variances of the means with the Student's *t*-test for unpaired samples.

Addition(s)	Cation content (nmol/mg)			
	Mg^{2+}	Δ [a]	Ca^{2+}	Δ [a]
None	26 ± 6	–	30 ± 10	–
EGTA, A23187	13 ± 3	–	6 ± 2	–
EDTA, A23187 [b]	11 ± 2	−2.0 [d]	4 ± 3	−1.3
EDTA	21 ± 3	−5.9 [c]	18 ± 9	−12.5 [c]
EDTA, P_i	22 ± 3	−4.7 [d]	11 ± 4	−19.5 [c]
EGTA, P_i	23 ± 4	−3.2	10 ± 4	−20.5 [c]

[a] Differences from untreated controls incubated with substrate only.
[b] Compared to EGTA + A23187 (S.D.).
[c] >95% confidence level.
[d] >80% confidence level.

brane permeability is more difficult with brain mitochondria.

EDTA, which by itself causes partial Na^+-dependent uncoupling (Tables I and II), lowered Mg^{2+} (by 6 nmol/mg) and also significantly decreased Ca^{2+} (by 12.5 nmol/mg; Table III). Phosphate, which clearly enhances uncoupling and presumably Na^+ conductance (Table I), did not significantly alter Mg^{2+} compared to EDTA alone (Table III). Although P_i lowered Ca^{2+}, extensive Ca^{2+} depletion alone is not sufficient to increase Na^+ permeability and uncoupling. Treatment with EGTA plus P_i, which does not uncouple, caused the same Ca^{2+} loss as EDTA and P_i (Table III).

These results indicated that if Mg^{2+} regulated Na^+ conductance, it must be a small pool (according to A23187 effects) and probably membrane bound (since EDTA was required under all conditions). Phosphate either acted independently of divalent cations or its mechanism was more complex (see Discussion).

In the course of these studies it was found that ruthenium red, an inhibitor of the Ca^{2+} permease [11,12] also inhibited Na^+ permeability [25]. These results are provided in Fig. 3A and B in comparison to Ca^{2+}-stimulated respiration as a measure of Ca^{2+} permeability.

Inhibition of uncoupling by Na^+ required approx. 150 pmol/mg ruthenium red (Fig. 3A) which was also the titer for comparable inhibition of passive swelling in NaCl, i.e., Na^+ conductance. Ruthenium red had no effect on swelling in sodium acetate, i.e. Na^+/H^+ exchange. Ca^{2+} conductance (State 3 respiration) was virtually abolished by 50 pmol ruthenium red or about one-third the level necessary to block Na^+ (Fig. 3A). This suggests Ca^{2+} and Na^+ permeabilities do not involve common pathways. These low titers for ruthenium red inhibition also suggested a limited number of sites or channels mediate Na^+ conductance and uncoupling.

EDTA is a relatively nonspecific chelator of various metal ions besides Mg^{2+} (and Ca^{2+}). The possible regulation of Na^+ conductance by cations other than Mg^{2+} was probed by testing chelators with ion selectivities different from those of EDTA. Na^+-dependent uncoupling was not obtained with triethanolamine or bathophenanthroline. Only slight uncoupling was observed with Na^+ and nitrilotriacetate or tetraethylenepentamine (tetran). However, Trien, which does not chelate Mg^{2+} [26], yielded about one-third the Na^+-dependent uncoupling of EDTA. The pattern of uncoupling with Trien was also qualitatively similar to EDTA as summarized in Table IV.

Phosphate increased uncoupling by Na^+ and Trien and could be replaced with A23187. Furthermore, Mg^{2+} or ruthenium red inhibited. However, uncoupling with Trien plus P_i was inhibited by EGTA presumably by chelating Ca^{2+}. This distinguishes Trien from EDTA which, of course, chelates Ca^{2+}. Trien not only does not chelate Mg^{2+} [26], but treating mitochondria with Trien under conditions sustaining uncoupling did not significantly alter endo-

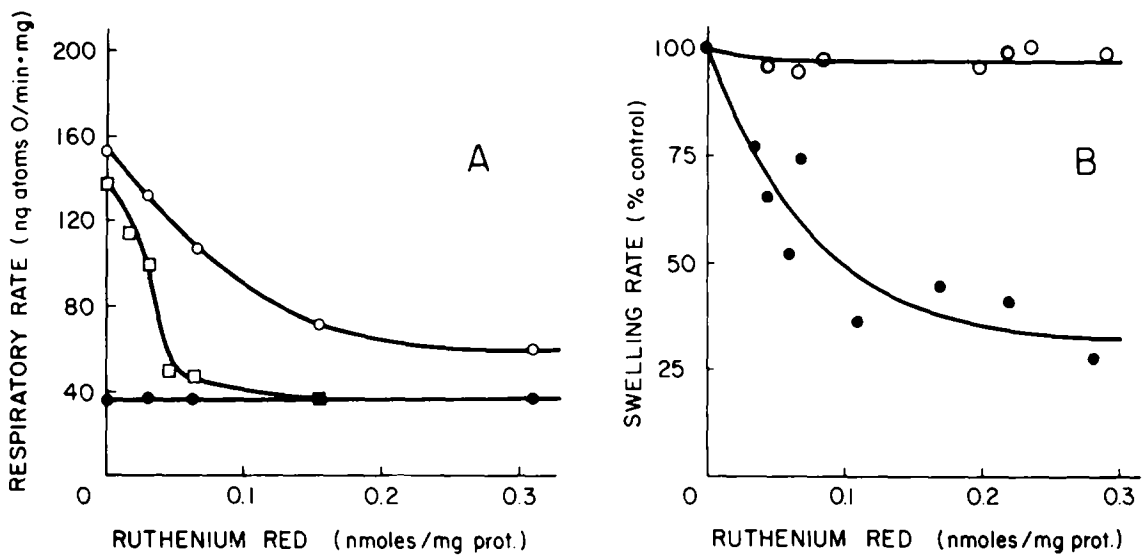

Fig. 3. (A) Mitochondria (0.6 mg/ml) were pretreated with rotenone (0.2 μg/mg). The effect of ruthenium red upon basal respiration rates with NaCl or choline chloride was determined with media containing 125 mM choline chloride or NaCl, 15 mM Tris-glycylglycine, 5 mM Tris-succinate, 2 mM Tris-P_i, 0.2 mM Tris-EDTA and 0.1% bovine serum albumin at pH 7.2 Ruthenium red inhibition of respiratory stimulation by Ca^{2+} (97 μM) was measured in choline chloride medium containing 1 mM $MgCl_2$ in place of EDTA.(○———○) NaCl, (□———□) Ca^{2+}, (●———●) choline chloride. (B) Passive mitochondrial swelling in 125 mM NaCl or sodium acetate at increasing ruthenium red concentrations was measured as described in the legend to Fig. 1 with mitochondria (0.15 mg/ml) pretreated with rotenone (0.2 μg/mg protein), suspended in medium containing 15 mM Tris-glycylglycine and 0.2 mM Tris-EDTA at pH 7.2 except without exogenous substrate or phosphate. (○———○) Sodium acetate, (●———●) NaCl.

TABLE IV

Na^+-DEPENDENT UNCOUPLING IN THE PRESENCE OF TRIEN

Mitochondria (0.6 mg/ml) pretreated with rotenone (0.2 μg/mg protein) were suspended in medium composed of 125 mM NaCl or choline chloride, 15 mM Tris-glycylglycine, 5 mM Tris-succinate, 0.1% bovine serum albumin and 1 mM Tris-Trien at pH 7.2. In addition, Tris-P_i (2 mM), $MgCl_2$ (1 mM), Tris-EGTA (0.2 mM), ruthenium red (0.03 mM), or A23187 (0.5 μg/mg), were included as indicated. The values are means (±S.D.) for at least three preparations.

Cation	Addition(s)	Respiratory rate (ngatom O/min per mg)
$Choline^+$	P_i	46 ± 6
Na^+	none	46 ± 2
Na^+	P_i	96 ± 17
Na^+	P_i, Mg^{2+}	64 ± 9
Na^+	P_i, ruthenium red	55 ± 9
Na^+	P_i, EGTA	58 ± 11
Na^+	A23187, EGTA	91 ± 23
$Choline^+$	A23187, EGTA	49 ± 2

genous mitochondrial Mg^{2+} or Ca^{2+} compared to controls (data not shown). The Na^+-dependent uncoupling with Trien may therefore involve a pathway other than that affected by EDTA.

Discussion

Certain observations and conclusions from these studies with brain mitochondria are in general agreement with those obtained with beef heart mitochondria by Brierley and co-workers [9,10,23,27–29]. Previous observations clearly indicated exogenous Mg^{2+} inhibited and EDTA stimulated Na^+ and K^+ conductances in brain [1] and heart [27] mitochondria. One could reasonably expect that endogeneous Mg^{2+} would behave similarly. Removing small amounts of endogeneous Mg^{2+} from heart mitochondria (approx. 1.0 nmol/mg protein and presumably bound) enhanced monovalent cation conductance [23]. Depleting brain mitochondria of only 2 nol Mg^{2+}/mg (the difference between A23187 plus EDTA and

EGTA plus A23187; Table III) is evidently necessary for cation conductance. However, these small differences are somewhat obscured by the high levels of bound Mg^{2+} and Ca^{2+}. Therefore this conclusion must be tentative.

The simplest explanation of Na^+ or K^+ uncoupling (stimulation of basal respiration; Fig. 1) is cation (Na^+ or K^+) and proton cycling. This involves inward conductance by an electrogenic uniport and efflux via neutral cation for H^+ exchange (reviewed in Refs. 9 and 10). These two pathways are also manifested in net cation uptake and swelling as opposed to cation release and contraction [30–35]. A clear dissociation of these two pathways is experimentally difficult [10]. One reason for this difficulty may be that the same or similar factors regulate each, e.g., Mg^{2+} [36, 37]. In studies with rat liver mitochondria, the intramitochondrial free [Mg^{2+}] evidently regulates neutral K^+/H^+ exchange [34,36,37]. Although subject to different interpretations, these observations argue against Mg^{2+} regulation of Na^+- or K^+-dependent uncoupling by preventing membrane 'stretching' and induction of H^+ 'leaks' [38].

The Na^+ conductance of brain mitochondria may be increased by A23187 or P_i by lowering the internal [Mg^{2+}] by depletion or complexation, respectively, as argued for neutral K^+/H^+ exchange [36,37]. This would explain why either can increase uncoupling by Na^+ although P_i does not lower the total mitochondrial Mg^{2+}. EDTA is necessary for maximal conductance under all conditions tested. This may be due to the high affinity of Mg^{2+} for external and internal regulatory sites of the inner membrane. One key question is which is the rate-limiting step in uncoupling, conductance or exchange? In the present and previous studies [1], we have maintained that conductance limits cation 'turnover' or cycling. This is based upon results with the model K^+ conductor, valinomycin [1], and effects such as those with ruthenium red. Ruthenium red inhibits passive swelling in NaCl (Na^+ conductance) and uncoupling yet it does not inhibit neutral Na^+/H^+ exchange (swelling in sodium acetate; Fig. 3A and B). However, instead of rates, the distinction may in fact be temporal. If net swelling via ionic conductance increases neutral exchange [33,34] until a steady state is achieved, then blocking conductance, e.g., with ruthenium red, prevents this from occurring and thereby uncoupling also. The present results with ruthenium red hold promise for further distinguishing between conductance and exchange pathways through direct determination of the number and affinity of the relevant ruthenium red-binding sites involved in conductance (nominally one per two electron-transport chains).

A physiological significance for Na^+ conductance is questionable, since it is inhibited by low [Mg^{2+}] [1,27]. However, an appreciable K^+ conductance exists in the presence of physiological levels of Mg^{2+} [1] and evidently exists in vivo according to the effects of K^+ upon respiration [39–41]. Hence, a K^+ conductance in vivo could be a factor in the rapid aerobic glycolysis of brain, e.g., via mitochondrial K^+ uptake, partial uncoupling and activation of pyruvate dehydrogenase [17]. Na^+ has been examined in greater detail in the present studies, since it is more amenable to the types of measurement employed here. Further study is required to identify definitely Na^+ and K^+ conductances with the same or different uniport systems in brain mitochondria.

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