Relationships between the Neuronal Sodium/Potassium Pump and Energy Metabolism

Effects of K^+, Na^+, and Adenosine Triphosphate in Isolated Brain Synaptosomes

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ABSTRACT The relationships between Na/K pump activity and adenosine triphosphate (ATP) production were determined in isolated rat brain synaptosomes. The activity of the enzyme was modulated by altering $[K^+]_e$, $[Na^+]_i$, and $[ATP]_i$ while synaptosomal oxygen uptake and lactate production were measured simultaneously. KCl increased respiration and glycolysis with an apparent K_m of about 1 mM which suggests that, at the $[K^+]_e$ normally present in brain, 3.3–4 mM, the pump is near saturation with this cation. Depolarization with 6–40 mM KCl had negligible effect on ouabain-sensitive O_2 uptake indicating that at the voltages involved the activity of the Na/K ATPase is largely independent of membrane potential. Increases in $[Na^+]_i$ by addition of veratridine markedly enhanced glycoside-inhibitable respiration and lactate production. Calculations of the rates of ATP synthesis necessary to support the operation of the pump showed that >90% of the energy was derived from oxidative phosphorylation. Consistent with this: (a) the ouabain-sensitive Rb/O_2 ratio was close to 12 (i.e., Rb/ATP ratio of 2); (b) inhibition of mitochondrial ATP synthesis by Amytal resulted in a decrease in the glycoside-dependent rate of ^{86}Rb uptake. Analyses of the mechanisms responsible for activation of the energy-producing pathways during enhanced Na and K movements indicate that glycolysis is predominantly stimulated by increase in activity of phosphofructokinase mediated via a rise in the concentrations of adenosine monophosphate [AMP] and inorganic phosphate [Pi] and a fall in the concentration of phosphocreatine [PCr]; the main moving force for the elevation in mitochondrial ATP generation is the decline in [ATP]/[ADP] [P_i] (or equivalent) and consequent readjustments in the ratio of the intramitochondrial pyridine nucleotides ($[NAD]_m/[NADH]_m$). Direct stimulation of pyruvate dehydrogenase by calcium appears to be of secondary importance. It is concluded that synaptosomal Na/K

pump is fueled primarily by oxidative phosphorylation and that a fall in [ATP]/
[ADP][P$_i$] is the chief factor responsible for increased energy production.

INTRODUCTION

The main function of the central nervous system (CNS), the generation, processing,
and transmission of impulses, depends on neuronal activity. Action potentials can be
initiated and synapses can transmit signals only if Na^+, K^+, and Ca^{2+} are maintained
in electrochemical disequilibrium across the plasma membrane. This situation
requires a constant input of energy and it is, therefore, not surprising that 40–50%
of the total adenosine triphosphate (ATP) produced in nervous tissue is utilized for
this purpose (Whittam, 1961; Baker, 1965; Ritchie, 1967; Michenfelder, 1974; Mata
et al., 1980; Astrup et al., 1981; Erecińska and Silver, 1989 for review). The key
enzyme involved in the maintenance of proper ion gradients is the Na/K pump
which mediates transport of 3 Na^+ outwards simultaneously with 2 K^+ inwards for
each ATP hydrolyzed (Skou 1965; Glynn and Karlish, 1975; Schuurmans-Stekhoven
and Bonting, 1981).

Synthesis of ATP in the CNS is accomplished by two processes: mitochondrial
oxidative phosphorylation and cytosolic glycolysis. The main fuel in brain is glucose,
and under aerobic conditions over 95% of it is oxidized to carbon dioxide and
water, with the balance being accounted for by the production of lactate (Siesjö,
1978; Erecińska and Silver, 1989). Thus mitochondria are responsible for over 95%
of the energy produced by the organ. In spite of this predominance of oxidative
phosphorylation, experiments with hippocampal slices have suggested that activa-
tion of the Na/K pump may be specifically dependent on glycolytically generated
ATP (Lipton and Robacker, 1983). This is not without a precedent because vascular
smooth muscle seems to rely on glycolysis for the maintenance of its Na^+ and K^+
gradients (Paul et al., 1979) and a similar situation has been reported to occur in the
cardiac myocyte (Weiss and Hiltbrand, 1985).

The regions of the CNS thought to be endowed with high rates of ion movements
are neuronal processes and synaptic terminals (Schwartz and Sharp, 1978; Kade-
karo et al., 1985). Consistent with this supposition, histochemical and immunocyto-
chemical studies have shown that activity of the Na/K ATPase is concentrated in the
plasma membrane of the above areas (Inomata et al., 1983; Pech and Stahl, 1984)
while biochemical investigations reported high velocities of ouabain-sensitive Na^+
and K^+-dependent ATP hydrolysis in isolated synaptosomes (Abdel-Latif et al.,
1970). Since it has been demonstrated that cytochrome c oxidase activity (the key
enzyme of the respiratory chain) is particularly abundant in dendrites and synaptic
terminals (Kageyama and Wong-Riley, 1982, 1986), it is logical to postulate that
oxidative phosphorylation is primarily responsible for providing metabolic energy to
support high rates of ion transport and ATPase activity.

To resolve this apparent controversy between the findings in vivo and those on
slices in vitro, the present study was undertaken to determine the relationships
between the Na/K pump activity and ATP production. Isolated nerve ending par-
ticles, synaptosomes, were used as a model because they retain a number of proper-
ties of CNS neurons and have been studied extensively in several laboratories
including our own (see Dagani and Erecińska, 1987, for references). Moreover, the

use of this preparation allows measurements to be done under a variety of well-controlled conditions in which influences from different contributing factors can be evaluated quantitatively and with high precision. Three specific questions were asked: (a) What are the proportions of ATP supplied by glycolysis and oxidative phosphorylation for the operation of the Na/K pump under resting and stimulated conditions? (b) What are the mechanism(s) responsible for activation of the energy producing pathways during increased ion pumping? (c) How efficient is glycolysis in supporting the Na/K pump activity when mitochondrial function is limited? The results presented in this work provide some answers to all three questions.

MATERIALS AND METHODS

Male Sprague-Dawley rats (220–250 g) were used throughout the study. Synaptosomes were isolated from the forebrains and midbrains as described by Booth and Clark (1978). In most experiments, the synaptosomal pellet was washed and suspended in a modified Krebs-Henseleit buffer (130 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 5 mM $NaHCO_3$) containing 10 mM Na-N-2-hydroxyethylpiperazine-N-2'-ethane sulfonic acid (HEPES), pH 7.4. In experiments in which the effect of potassium was studied the concentration of KCl was lowered to 0.25 mM.

Incubation Conditions

Synaptosomes were suspended at either 3–4 mg protein/ml (measurements of oxygen uptake, metabolite levels, and pyruvate dehydrogenase activity) or 1 mg protein/ml (measurements of 2-deoxyglucose and ^{86}Rb uptake) and incubated for 10 min at 30°C in a shaking water bath. In most experiments, the suspension was supplemented with 10 mM glucose and 1.27 mM $CaCl_2$. The former was deleted for measurements of pyruvate dehydrogenase activity and lowered to 0.5 mM in determinations of 2-deoxyglucose uptake. When the effect of calcium elimination was studied, $CaCl_2$ was omitted and replaced with 10 mM $MgSO_4$. All samples taken after 10 min of preincubation are referred to throughout the text as time 0.

Oxygen Uptake and Metabolite Levels

Synaptosomes preincubated as above were further incubated at 30°C in a shaking water bath for either 1, 5, 15, or 30 min with and without the desired concentrations of the test compounds. Aliquots were withdrawn and used either for the measurement of O_2 uptake or rapidly quenched by addition of cold perchloric acid (0.6 M final concentration). Oxygen uptake was assayed with a Clark-type electrode in a stirred chamber thermostated at 30 ± 1°C. The perchloric acid extracts were neutralized with 2.5 M $KHCO_3$ and centrifuged, and the clear supernates were used for the determination of metabolites by standard enzymatic techniques (Bergmeyer, 1974; and Dagani and Erecińska, 1987 for details).

Uptake of 2-Deoxyglucose

Uptake studies were initiated by addition of 20 nM (0.5 μCi/ml) 2-[1,2-^{3}H]deoxy-D-glucose (2-DG; 42 Ci/mmol, New England Nuclear, Boston MA). When appropriate, ouabain was added 1 min before 2-DG whereas all other test compounds were supplied at time 0. Aliquots were withdrawn after 0.5, 1.5, 3, 6, and 9 min and rapidly centrifuged through a layer of silicone oil (1.03 sp gr, General Electric Co., Waterford, NY). Radioactivity was then counted in the pellets and total suspensions in a Searle Delta 300 liquid scintillation counter (T.M. Analytic, Elk Grove Village, IL) using Liquiscint 121 (National Diagnostics, Sommerville, NJ).

Rates of 2-DG uptake were calculated by linear regression analysis of the increase in radioactivity in the pellets.

Uptake of ^{86}Rb

Uptake of ^{86}Rb was measured by the method of Scott and Nicholls (1980) in a medium containing 5 mM KCl, unless indicated otherwise. After preincubation, aliquots were transferred to 25-ml Erlenmeyer flasks containing various test compounds and 50 μM ^{86}RbCl (0.3 μCi/ml; ^{86}Rb, 20 Ci/g, New England Nuclear). Samples (350 μl) were taken at 0.5, 1, 2.5, and 30 min and centrifuged through silicone oil as described above. Radioactivity was then counted in the pellets, supernatants and total suspensions. Rates of ^{86}Rb uptake were calculated by linear regression analysis of the increase in radioactivity in the pellets. The final rubidium accumulation was estimated from the counts present in the pellets and supernatants after 30 min of incubation. Intracellular water was estimated to be 4 μl/mg protein (Pastuszko et al., 1981).

Pyruvate Decarboxylation

At the end of the preincubation the synaptosomal suspensions were supplemented with 0.2 μCi/ml of either [1-^{14}C]- or [3-^{14}C]pyruvate (15 and 20 mCi/mmol, respectively, New England Nuclear) and the required concentrations of the appropriate test compounds. The final incubation volume was 0.5 ml. The tubes were sealed with rubber stoppers equipped with plastic center wells. At the desired intervals, the incubations were terminated by injection of 0.1 ml of 35% trichloroacetic acid (TCA) into the sealed tubes. The $^{14}CO_2$ liberated was trapped by adding 0.2 ml of 10% KOH to the center well. The tubes were agitated at room temperature for 1 h before the center wells were removed to scintillation vials containing 10 ml of Liquiscint 121. The rate of pyruvate decarboxylation presented in nanomoles per minute per milligram of protein was corrected for nonenzymatic decarboxylation of ^{14}C-labeled pyruvate by measuring the rate of $^{14}CO_2$ production in the absence of synaptosomes.

Measurements of Cytosolic Free Calcium Using Quin 2

The levels of cytosolic free calcium were measured with the fluorescent probe Quin 2 as described by Tsien et al. (1982) and adapted for synaptosomes by Hansford and Castro (1985) with some modifications. The synaptosomal pellet was suspended at about 2 mg protein/ml in Krebs-Henseleit-HEPES buffer, pH 7.4, supplemented with 1.27 mM calcium and 10 mM glucose and maintained at 37°C. After 10 min of incubation with shaking at 37°C, Quin 2/AM (Sigma Chemical Co., St. Louis, MO) dissolved in dimethyl sulfoxide (5 mM stock solution) was added slowly to a final concentration of 50 μM. The suspension was further incubated for 45 min under the same conditions. All subsequent steps were performed as given by Hansford and Castro (1985). The fluorescence of Quin 2-calcium complex was recorded in a spectrofluorometer (model LS5, Perkin-Elmer Corp, Norwalk, CT). The excitation wavelength was 339 nm (5-nm bandwidth) and the emission 395 nm (10-nm bandwidth). Synaptosomes suspended in the medium specified above (1 mg protein/ml) were placed in a 3-ml cuvette thermostated at 30°C and stirred throughout the recording with a small magnetic bar. No difference was observed when the recordings were made at 37°C. The calibration of calcium concentrations was carried out as given by Tsien et al. (1982) and Capponi et al. (1986).

Other Measurements

Protein was determined by the biuret reaction using bovine serum albumin as the standard.

RESULTS

Potassium and Synaptosomal Respiration

The effects of K^+ on respiration were investigated using two experimental protocols. In the first, synaptosomes were supplemented with the required concentration of KCl and incubated for 30 min, and then their respiration was measured. In the second, preparations were preincubated for 10 min and transferred to the oxygen electrode chamber, and the desired [KCl] was added while the respiratory activity was monitored continuously for the next 5–7 min. In both protocols KCl in the incubation media was varied by replacement with equimolar quantities of NaCl or choline chloride, so that the osmolality of all media was the same. Neither had any effect in the concentration range 1–40 mM on synaptosomal respiration either in the long-term (30 min of incubation) or short-term (addition in the oxygen electrode chamber) protocol.

In the complete absence of K^+ in the medium O_2 uptake was 2.68 ± 0.5 nmol/min per mg protein, somewhat higher than the value of 2.43 ± 0.4 nmol/min per mg protein obtained with 1 mM ouabain. To avoid the possibility that this enhanced respiration at 0 external potassium was due to membrane depolarization seen under such conditions (Burke et al., 1988), synaptosomes for this series of experiments were isolated and suspended in the presence of 0.25 mM KCl. Because of the difficulty in establishing the true level of respiratory activity in the complete absence of external K^+, the contribution of the Na/K pump to oxygen uptake was defined as the ouabain-sensitive portion of the total.

The concentration of ouabain used in this study was 1 mM and it gave rapid and maximal reduction in O_2 uptake. Although the rat cerebral Na/K pump is inhibited by ouabain with a concentration for 50% inhibition ($K_{0.5}$) of about 1 μM (Shirachi et al., 1970; Urayama and Nakao, 1979; Akera et al. 1985; Sweadner, 1985) and this value is rather insensitive to addition of external K^+ (Urayama and Nakao, 1979; Akera et al., 1985; Sweadner, 1985), a large concentration of the glycoside was used to ensure complete blockage of the pump even at increased [KCl]. Under the conditions used, respiration inhibition with ouabain was very consistent from preparation to preparation and stable over 30 min of incubation.

Fig. 1 *A* shows that O_2 consumption in synaptosomes incubated for 30 min was stimulated in a dose-dependent manner by increasing concentrations of KCl. The maximal effect was attained with 3–6 mM KCl while at 40 mM KCl a decline from the plateau value was observed. Addition of 1 mM ouabain decreased the respiratory activity by about 30% of that observed with 3–6 mM KCl. Taking the ouabain-inhibited rate as that corresponding to 0 KCl, the $K_{0.5}$ for stimulation of respiration was calculated to be 1.8 mM (Fig. 1 *B*).

Essentially the same titration curve was obtained when 5 mM pyruvate, instead of glucose, was used as the substrate except that O_2 uptake was 30–40% higher. This increase in respiration is due to activation of flux through pyruvate dehydrogenase by a high level of pyruvate and is consistent with previous studies of other investigators (Booth and Clark, 1978; Kauppinen et al., 1986). At 3–10 mM KCl, respiration was 4.6–4.9 nmol/min per mg protein and it was 33% inhibited by 1 mM ouabain (data not shown).

Different responses were seen when immediate effects of K^+ were followed. With 1–6 mM KCl gradual increases in respiration were observed which gave values not dissimilar to those after 30 min incubation. However, at [KCl] higher than 6 mM, marked biphasic behavior was noted: an initial rapid stimulation of respiration, the magnitude of which rose with the increase in [KCl], was followed by a swift decline to a new steady state (Fig. 2, trace A). In 15 min this steady state was not different from that seen in long-term incubations. The initial rapid rise in oxygen consumption was eliminated by addition of 2 mM Amytal (Eli Lilly & Co., Indianapolis, IN; not shown) and almost completely abolished by the removal of calcium from the medium (and simultaneous addition of 10 mM Mg^{2+}, Fig. 2, trace B) but was unaffected by the presence of ruthenium red (Fig. 2, trace C), a known inhibitor of
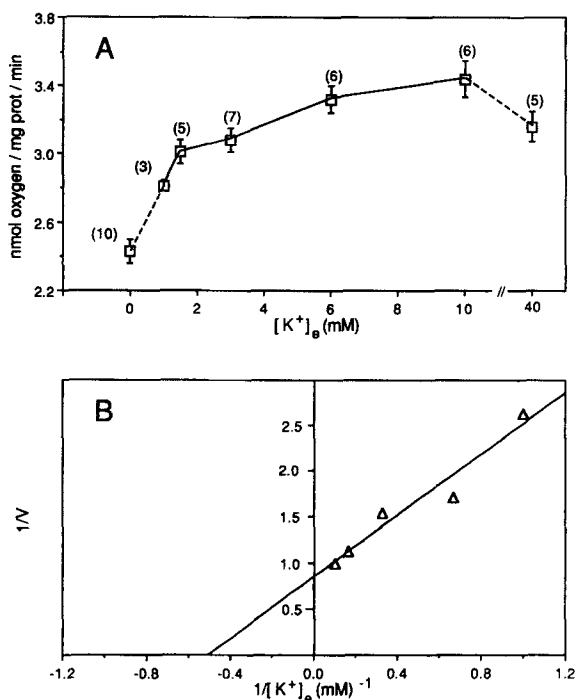

FIGURE 1. Stimulation of synaptosomal respiration by $[K^+]_e$. (A) Respiration was measured as described in Materials and Methods on synaptosomes incubated for 30 min in a modified Krebs-Henseleit buffer, pH 7.4, containing [KCl] indicated in the figure. O_2 uptake marked as 0 mM KCl is that in a medium with 0.25 mM KCl and 1 mM ouabain. Values represent means ± SEM for the number of experiments in parentheses. (B) Double reciprocal plot of the KCl-stimulated minus 0 KCl rates of oxygen uptake against [KCl]. Experimental points are from A. The solid line represents computer-derived best fit to the data points.

calcium entry into the mitochondrion both in isolated organelles (Moore, 1971) and in synaptosomes (Hansford and Castro, 1985). In independent experiments, the same concentration of ruthenium red completely blocked Ca^{2+}-induced enhancement of oxygen uptake in mitochondria isolated from liver and brain.

The stimulation of O_2 consumption caused by addition of low [KCl] (1–6 mM) in short-term experiments was, to a large extent, inhibited by pretreatment with 1 mM ouabain whereas it was much less sensitive at $[K^+]$ higher than 6 mM. This suggests that the rapid increase in respiration that follows the addition of greater amounts of KCl is not related to activation of the Na/K pump. Since [KCl] above 5–6 mM is known to depolarize the synaptosomes (Blaustein and Goldring, 1975) titrations with KCl of O_2 uptake were carried out in the presence and absence of ouabain. The
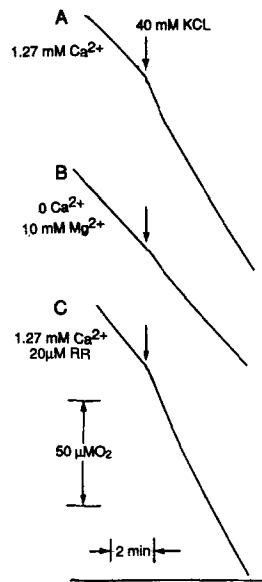
FIGURE 2. Effect of calcium withdrawal and addition of ruthenium red (RR) on stimulation of respiration caused by 40 mM KCl. O_2 uptake was measured as given in Materials and Methods after a 10-min preincubation of synaptosomes in media modified as indicated in the figure. Protein concentration was 3.52 mg/ml.
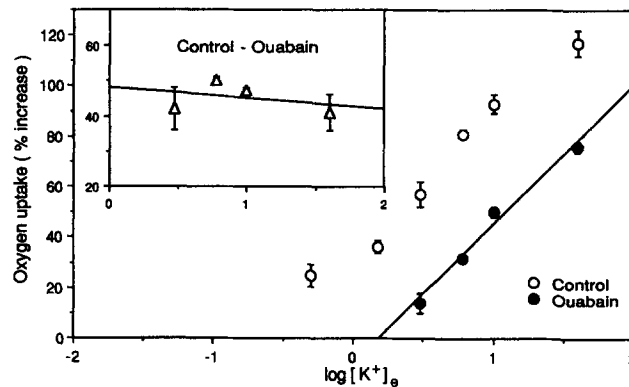

FIGURE 3. Rates of oxygen uptake by synaptosomes incubated with and without ouabain at various $[K^+]_e$. Synaptosomes were preincubated for 10 min in a medium containing 0.25 mM KCl and either 0 or 1 mM ouabain. O_2 uptake recorded at the end of the preincubation was considered as the basal rate. KCl, at concentrations specified in the figure, was then added (see Fig. 2 A) and respiratory activity measured by drawing the tangent to the initial rise in oxygen consumption. The results were calculated as percentages of increases in respiration above the basal rate for each [KCl] and represent means ± SEM for four experiments. Mean basal rate without ouabain was 2.73 ± 0.03 nmol/min per mg protein (±SEM, $n = 22$) and 2.59 ± 0.02 ($n = 16$) in its presence. The inset shows the plot of differences in respiration with and without ouabain against log [KCl]. The solid lines are computer-derived linear best fits to the data.

results are plotted in Fig. 3 as percentages of stimulation of respiration against the log of [K$^+$] in the external medium ([K$^+$]$_e$). It can be seen that in the absence of the glycoside the curve was composed of two parts. There was an initial increase in respiration by about 30–40% which reached a plateau at ~3 mM KCl. This was followed at a concentration of about 5–6 mM by an almost linear rise, with no apparent signs of saturation at 40 mM KCl. The curve with ouabain lacked the initial increase at low [KCl] and was a straight line, parallel to the second portion of the titration without glycoside. When values obtained in the presence of ouabain were subtracted from those in its absence, a straight line was obtained (inset, Fig. 3) at a value of ~40% increase in respiration and a slope not significantly different from 0. Because in synaptosomes membrane potential is the K$^+$ diffusion potential (Blaustein and Goldring, 1975) and assuming that ouabain-sensitive O$_2$ uptake at various [K$^+$] reflects the Na/K pumping rate our results would suggest that the amount of ATP utilized for this function in intact synaptosomes is not detectably affected by changes in membrane potential.

Potassium and Synaptosomal Lactate Production

The rates of lactate production were measured under aerobic conditions after 30 min of incubation with glucose in samples parallel to those used for the measurement of O$_2$ uptake. The results presented in Fig. 4 A show that the amount of lactate formed increased with a rise in [KCl] and reached a plateau at 3 mM KCl. With 40 mM KCl, less lactate was generated than with 3–6 mM KCl. Maximal rates of synthesis were 0.89 ± 0.07 nmol/min per mg protein and they were 50% inhibited (to 0.47 ± 0.04 nmol/min per mg protein) by 1 mM ouabain. When the amount of lactate formed in the presence of the glycoside was taken to correspond to that with 0 KCl, the $K_{0.5}$ for stimulation by K$^+$ of lactate formation was calculated to be 0.7 mM. This means that the increase in glycolysis associated with the Na/K pump activity occurs with a $K_{0.5}$ for K$^+$ of ~1 mM.

When RbCl was used to stimulate the Na/K pump the responses observed were the same as those with KCl displayed in Figs. 1 and 3 (not shown). Lactate production was also increased with a rise in rubidium concentration and reached saturation at the same value as that seen with KCl.

Activity of the Synaptosomal Na/K Pump

To estimate directly the activity of the Na/K pump, the rates of ^{86}Rb transport were measured in the presence of 1 mM and 5 mM KCl in a medium containing 50 μM [86]RbCl. With 1 mM KCl, the velocity of ^{86}Rb influx was 12.93 ± 1.5 nmol/min per mg protein (mean ± SEM, $n = 4$) and it declined to 7.68 ± 0.78 nmol/min per mg protein (mean ± SEM, $n = 4$) with 1 mM ouabain; thus the ouabain-sensitive uptake amounted to 5.25 ± 0.58 nmol/min per mg protein. The corresponding values with 5 mM KCl were 17.45 ± 0.47, 7.65 ± 0.26, and 9.8 ± 0.26 nmol/min per mg protein (see Table V below). (The same rates were obtained when KCl was replaced by RbCl.) Using the value of 0.89 nmol/min per mg protein (3.32 at 6 mM KCl − 2.43 with 1 mM ouabain, Fig. 1) for the ouabain-sensitive portion of O$_2$ consumption and the value of 9.8 nmol/min per mg protein for the glycoside-sensitive rubidium
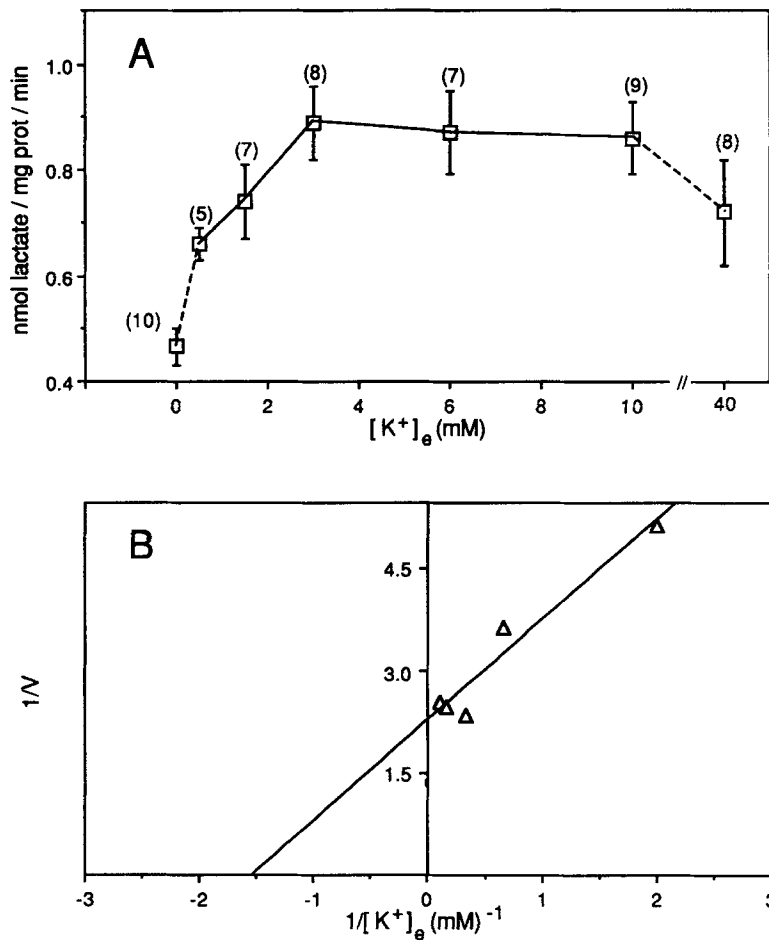
FIGURE 4.   Stimulation by potassium of lactate production in suspensions of rat brain synaptosomes. (A) Lactate was measured in incubations identical to those in Fig. 1 A. Values represent means ± SEM for the number of experiments in parentheses. (B) Double reciprocal plots of K^+-induced increases in lactate production against $[K^+]_i$. Data points are from A. The solid line is the best fit to the data.

uptake one can calculate a Rb/O_2 ratio of 11.5 at 5 mM KCl. This corresponds to an Rb/ATP ratio of 2.

Effect of Veratridine on Synaptosomal Oxygen Uptake and Lactate Production

The consequences of stimulation of the Na/K pump by sodium on synaptosomal ATP production were investigated using veratridine to raise the internal concentration of the cation. This alkaloid opens the voltage-sensitive Na^+ channels and causes influx of sodium and membrane depolarization (Ulbricht, 1969). The results are summarized in Tables I and II and Fig. 5. Preliminary experiments had established

TABLE I

O_2 Uptake by Synaptosomes Incubated with and without 1.27 mM Ca^{2+}: Effects of Mg^{2+} (10 mM), Ouabain (1 mM), and Veratridine (50 μM)

Condition	Incubation time		
	min		
	0	5	30
	nmol/mg protein per min		
Ca^{2+}	3.71 ± 0.05 (13)		3.65 ± 0.08 (4)
Mg^{2+}	3.31 ± 0.06 (9)*		3.18 ± 0.03 (5)*
Ca^{2+} + ouabain	2.93 ± 0.07 (8)*‡		2.87 ± 0.03 (3)*‡
Mg^{2+} + ouabain	2.35 ± 0.02 (3)*‡§		2.31 ± 0.01 (3)*‡§
Veratridine			
Ca^{2+}		9.20 ± 0.14 (6)ǀ	5.41 ± 0.35 (6)ǀ¶
Mg^{2+}		8.67 ± 0.20 (3)ǀ	7.12 ± 0.03 (3)*ǀ¶
Ca^{2+} + ouabain		3.37 ± 0.06 (5)*‡§ǀ	2.70 ± 0.06 (5)*‡¶
Mg^{2+} + ouabain		2.89 ± 0.06 (2)*‡ǀ	2.59 ± 0.1 (2)*‡¶

Experimental conditions are given in Materials and Methods. Results are means ± SEM for the number of experiments in parentheses. *Different from Ca^{2+}; ‡different from Mg^{2+}; §different from Ca^{2+} + ouabain; ǀdifferent from zero time; ¶different from 5-min incubation (one-way ANOVA and Tukey test). All P values were <0.05 or less.

that 25–50 μM veratridine gave maximal effects. Hence, 50 μM veratridine was used in all subsequent studies.

The results show that veratridine caused a marked increase in respiration. Withdrawal of calcium and simultaneous addition of 10 mM magnesium lowered the respiratory activity only very slightly. The rates of oxygen uptake were constant over 30

TABLE II

Lactate Production by Synaptosomes Incubated with and without 1.27 mM Ca^{2+}: Effects of Mg^{2+} (10 mM), Ouabain (1 mM), and Veratridine (50 μM)

Condition	Time	
	min	
	15	30
	nmol/mg protein per min	
Ca^{2+}	0.76 ± 0.05 (4)	0.85 ± 0.07 (4)
Mg^{2+}	0.87 ± 0.04 (3)	0.95 ± 0.08 (3)
Ca^{2+} + ouabain	0.28 ± 0.05 (3)*‡	0.40 ± 0.00 (3)*‡
Mg^{2+} + ouabain	Not measured	Not measured
Veratridine		
Ca^{2+}	1.67 ± 0.08 (6)§	1.68 ± 0.66 (6)§
Mg^{2+}	2.82 ± 0.10 (3)*§	2.96 ± 0.12 (3)*§
Ca^{2+} + ouabain	0.35 ± 0.04 (5)*‡	0.35 ± 0.05 (5)*‡
Mg^{2+} + ouabain	0.26 ± 0.17 (2)*‡	0.40 ± 0.00 (2)*‡

Conditions are given in Materials and Methods. Values are means ± SEM for the number of experiments in parentheses. Statistical evaluation was performed using one-way ANOVA and Tukey test. *Different from Ca^{2+}; ‡different from Mg^{2+}; §different from control. All values at 30 min of incubation were significantly different from those at 15 min. P values in all cases were <0.05 or less.

min of incubation in control samples under all conditions investigated. However with veratridine, a decline over time was observed when calcium was present in the medium. Ouabain had a dramatic effect on veratridine-stimulated respiratory activity: it reduced it to a level which was not much higher than that in glycoside-treated control samples. By contrast, ruthenium red did not affect the stimulation of respiration caused by veratridine (Fig. 5). Essentially the same behavior was observed when 5 mM pyruvate instead of glucose was used as the respiratory substrate: O_2
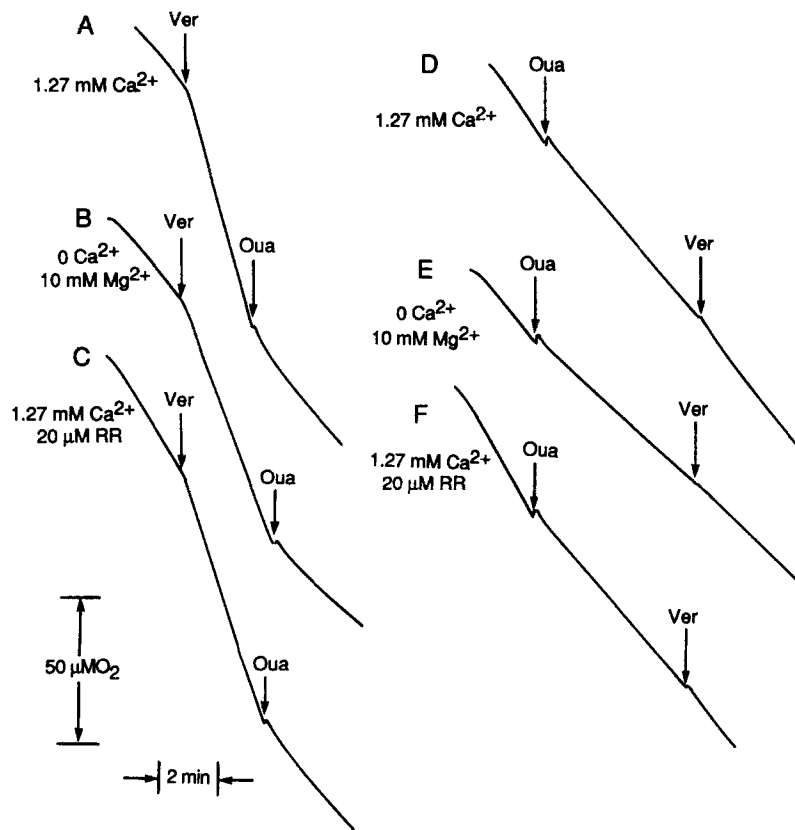
FIGURE 5. Effect of calcium withdrawal, ruthenium red (RR), and ouabain (Oua) on veratridine (Ver)-induced increase in respiration. O_2 uptake was measured under conditions shown in the figure after a 10-min preincubation. Protein was 3.12 mg/ml, veratridine 50 μM, and ouabain 1 mM.

uptake was stimulated about threefold by veratridine but this was inhibited almost completely by ouabain.

The rates of lactate production were also markedly enhanced by veratridine. Calcium suppressed glycolytic activity somewhat: its influence was greatest in samples incubated with veratridine but without ouabain. Veratridine increased by twofold the amount of lactate formed in the presence of Ca^{2+} and by threefold in its absence; the increases were completely eliminated by 1 mM ouabain. Consequently,

the ouabain-sensitive rates of lactate generation were enhanced by veratridine three-to sixfold.

To confirm the enhancement of the glycolytic flux as measured by lactate production, uptake of 2-DG, a substrate for the glucose transporter in synaptosomal plasma membrane (Diamond and Fishman, 1973) was determined under the same experimental conditions. The results obtained were consistent with those derived from estimates of lactate levels. The basal rate of 2-DG uptake was 15.62 ± 1.06 fmol/min per mg protein and decreased to 8.38 ± 0.61 fmol/min per mg protein (mean $\pm$ SEM, $n = 5$; $P < 0.001$) in the presence of 1 mM ouabain and increased to 46.3 ± 4.15 fmol/min per mg protein (mean $\pm$ SEM, $n = 4$; $P < 0.001$ with respect to control) in the presence of 50 μM veratridine. When both ouabain and veratridine were added, 2-DG transport was not significantly different from that with ouabain only (10.05 ± 1.53 fmol/min per mg protein, $n = 4$). (Statistical analyses were performed using one-way analysis of variance [ANOVA] plus Tukey test.)

Effect of Na/K Pump Stimulation on Synaptosomal ATP, Phosphocreatine (PCr), and Creatine (Cr)

The intrasynaptosomal levels of ATP, PCr, and Cr were measured after 5 and 30 min of incubation with veratridine and with or without ouabain. Decreases in PCr and ATP and increases in Cr were observed in the presence of veratridine which were already detectable after 5 min of incubation (Table III). These changes were prevented, to a large extent, by addition of ouabain.

Rates of Pyruvate Decarboxylation by Synaptosomes

Pyruvate is the key respiratory substrate formed in synaptosomes during the metabolism of glucose. For this reason, the rates of its decarboxylation were measured using 1-^{14}C- and 3-^{14}C-labeled pyruvate. $^{14}CO_2$ produced from the former arises from two reactions: the conversion to acetylcoenzyme A via pyruvate dehydrogenase and by the combined action of enzymes of the TCA cycle and the gluconeogenic enzyme phosphoenolpyruvate carboxykinase. We have confirmed (unpublished data) the results of Shank et al. (1985) that gluconeogenic enzymes are absent from synaptosomes; hence decarboxylation of $[1$-^{14}C]pyruvate reflects the operation of the pyruvate dehydrogenase complex. $^{14}CO_2$ formed from $[3$-^{14}C]pyruvate measures decarboxylation catalysed primarily by enzymes of the TCA cycle (Claus and Pilkis, 1977).

Results of the determinations carried out for 5 and 30 min with the two radioactive pyruvates are presented in Table IV. It can be seen that veratridine stimulated the rate of $[1$-^{14}C]pyruvate decarboxylation twofold in 5 min and by ~45% after 30 min. The smaller stimulation after longer incubation is consistent with the lesser enhancement of O_2 uptake observed under the same conditions (Table I). The stimulation of pyruvate dehydrogenase activity was almost completely prevented by ouabain (Table I) but was unaffected by ruthenium red (Fig. 5). Surprisingly, after longer incubations ruthenium red even increased the rate of $[1$-^{14}C]pyruvate decarboxylation, especially with veratridine present.

Essentially the same behavior was observed in experiments with $[3$-^{14}C]pyruvate, except that the absolute rates were much smaller while stimulation by veratridine

TABLE III

Effects of KCl (40 mM) and Veratridine (50 μM) on Synaptosomal ATP, Phosphocreatine, and Creatine

Addition	ATP		PCr		Cr	
Incubation time (*min*)	5	30	5	30	5	30
			nmol/mg protein			
None (control)	4.13 ± 0.31	3.86 ± 0.39	6.95 ± 0.58	6.66 ± 0.45	14.53 ± 1.27	15.87 ± 0.89
Veratridine	3.21 ± 0.37*	2.86 ± 0.41*	4.15 ± 0.14*	3.84 ± 0.27*	18.83 ± 0.77	18.43 ± 0.81*
Veratridine + 1 mM ouabain	3.90 ± 0.49‡	3.66 ± 0.32‡	6.40 ± 0.41‡	6.15 ± 0.38‡	16.73 ± 0.20	16.98 ± 0.28

Conditions of incubation and methods of analysis are given in Materials and Methods. Values are means ± SEM for three experiments. *$P < 0.05$ with respect to control; ‡$P < 0.05$ with respect to veratridine. Statistical evaluation was performed using Student's t test for paired data.

was larger. This apparent enhancement of $^{14}CO_2$ evolution in the presence of the alkaloid may be caused by a lesser dilution of the pool of the TCA cycle intermediates by nonlabeled substrates produced by other metabolic reactions. The most prominent of those is 2-oxoglutarate which is formed in the nerve endings by avid metabolism of glutamate (Erecińska et al., 1988). During depolarization, glutamate is rapidly released from synaptosomes which reduces the supply of 2-oxoglutarate and thus diminishes its impact on the oxidation of [3-^{14}C]pyruvate.

Measurements of Intrasynaptosomal Calcium Concentration

Although levels of cytosolic free calcium in synaptosomes have been measured by several investigators and under a variety of conditions (see e.g., Ashley et al., 1984; Richards et al., 1984; Hansford and Castro, 1985; Brethes et al., 1987), we have

TABLE IV

Rates of [^{14}C] Pyruvate Oxidation by Synaptosomes: Effects of Veratridine (50 µM), Ruthenium Red (20 µM) and Ouabain (1 mM)

Additions Incubation time (min)	[1-^{14}C]pyruvate		[3-^{14}C]pyruvate	
	5	30	5	30
		nmol/mg protein		
None	13 ± 0.51	64.4 ± 5.3	0.24 ± 0.04	3.50 ± 0.29
	(5)	(4)	(5)	(4)
Ruthenium red	13.7 ± 0.56	70.8 ± 6.7	0.45 ± 0.1	6.0 ± 0.49
	(4)	(4)	(4)	(4)
Veratridine	26.2 ± 1.20*	93.2 ± 4.9*	1.64 ± 0.28*	28.4 ± 3.64*
	(5)	(4)	(5)	(4)
Ruthenium red + veratridine	23.7 ± 1.08*	110.8 ± 8.9*	1.95 ± 0.33*	31.0 ± 3.33*
	(4)	(4)	(4)	(4)
Ouabain	12.5 ± 0.71	ND	0.2 ± 0	ND
	(2)		(2)	
Ouabain + veratridine	15.1 ± 0.58‡	ND	0.33 ± 0.14‡	ND
	(3)		(3)	

Experimental conditions are given in Materials and Methods. Values are means ± SEM for the number of experiments in parenthesis. *Significantly different with respect to no addition at P values of 0.01 or less. ‡Significantly different from veratridine at P < 0.001 (Student's t test for two means). ND, not determined.

repeated these studies to allow precise correlation with other determinations performed in this work. The [Ca$^+$]$_{free}$ was found to be 256 ± 12 nM (mean ± SEM, n = 10) and it was increased by KCl, veratridine and ouabain as expected (Ashley et al., 1984). The kinetics of responses to various stimuli were quite different. KCl at concentrations higher than 6 mM caused a dose-dependent rise in [Ca^{2+}]$_i$ which peaked within 5–10 s and declined to a baseline value after 4–6 min. With 40 mM KCl the intrasynaptosomal concentration of this cation was found to be 395 ± 12 nM. Veratridine at 50 µM induced a much larger increase (to 563 ± 47 nM) which required 4–6 min to attain a maximum. Ouabain addition resulted in a slow, gradual rise which did not reach a plateau during the 6–8 min of the recording. The glycoside administered before either 40 mM KCl or 50 µM veratridine did not affect subsequent increase in [Ca^{2+}]$_i$ caused by these agents.

Effect of Energy Level on the Activity of the Na/K Pump

Concentration of ATP in synaptosomes can be effectively lowered by the addition of respiratory chain inhibitors. Fig. 6 shows, in agreement with our earlier studies (Dagani and Erecińska, 1987), that Amytal at 1 mM inhibited oxygen uptake by ~30% and at 2 mM by ~70%. Over 80% blockage was obtained with 10 μM rotenone. Concomitantly, the level of ATP decreased from 3.86 ± 0.39 to 3.14 ± 0.23, 1.59 ± 0/15, and 0.82 ± 0.06 nmol/mg protein (mean ± SEM for $n = 3$) in the presence of 1, 2 mM Amytal and 10 μM rotenone, respectively after 30 min of incu-
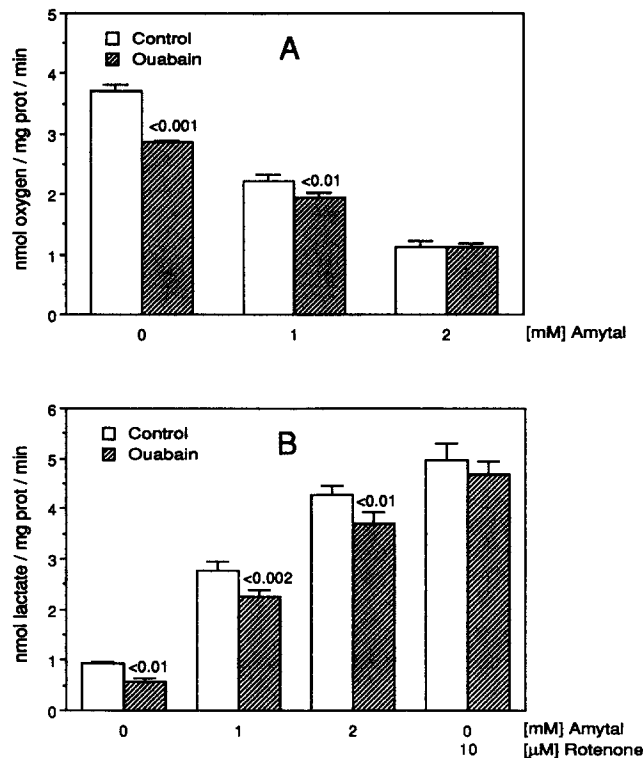

FIGURE 6.   Effect of ouabain on respiration and lactate production by Amytal or rotenone-treated synaptosomes. Synaptosomes were incubated for 30 min either with or without the test compounds and their O_2 uptake (A) and lactate production (B) measured as described in Materials and Methods. Values represent means ± SEM for six experiments.

bation. This was accompanied by a rise in lactate production from 0.93 ± 0.03 nmol/mg protein per min in control samples to a maximum rate of 4.97 ± 0.3 nmol/mg protein per min with rotenone present (Fig. 6). However, surprisingly neither the residual respiration nor the increased rates of lactate production in the presence of the inhibitors of the mitochondrial respiratory chain were very sensitive to addition of ouabain. With 1 mM Amytal, the glycoside blocked lactate synthesis by ~20%, with 2 mM Amytal by ~15% and with rotenone by 6% (Fig. 6). With 2 mM Amytal and 10 μM rotenone the rates of lactate production were not stimulated

further by addition of 50 μM veratridine and they were the same with and without calcium in the medium.

To explore the possibility that a decrease in the level of ATP would limit activity of the Na/K pump, rates of ^{86}Rb uptake were measured with and without ouabain. Table V shows that, in the presence of 1 mM Amytal, the ouabain-sensitive rate of ^{86}Rb uptake was already inhibited by 35%. The inhibition was increased to 65% with 2 mM Amytal. When glucose was omitted in the presence of 2 mM Amytal and 1 mM ouabain, the rate of ^{86}Rb influx decreased to <10% of its original value.

The decrease in activity of the Na/K pump could lead to the collapse of ion gradients and consequent depolarization of the synaptosomal plasma membrane. For this reason, transmembrane electrical potential was estimated from the distribution of ^{86}Rb in preparations incubated for 30 min under conditions listed in Table V. Using the Nernst equation (59 log $[Rb^+]_i/[Rb^+]_e$) the values calculated were -57 ± 1.3 mV for control, -54 ± 1.2 mV with 1 mM Amytal, -51 ± 1.1 mV with 2 mM Amytal, and -43 ± 2.2 mV with 2 mM Amytal and no glucose. These were consis-

TABLE V

Rates of Ouabain-sensitive ^{86}Uptake by Synaptosomes Treated with Amytal

Additions Amytal	Glucose	^{86}Rb uptake		
		-1 mM ouabain	$+1$ mM ouabain	Ouabain-sensitive
mM	*mM*	*nmol/mg protein per min*		
0	10	17.45 ± 0.47	7.65 ± 0.26	9.80 ± 0.26
1	10	$13.65 \pm 0.63*$	7.40 ± 0.28	$6.25 \pm 0.35^{\ddagger}$
2	10	$11.43 \pm 0.75^{\ddagger}$	7.95 ± 0.98	$3.47 \pm 0.43^{\ddagger}$
2	0	$8.53 \pm 0.64^{\ddagger}$	7.77 ± 0.57	$0.77 \pm 0.32^{\ddagger}$

Experimental conditions and details of measurements are given in Materials and Methods. Values are means ± SEM for three (1 mM Amytal) or four (0 and 2 mM Amytal) experiments. *$P < 0.005$; $^{\ddagger}P < 0.001$ with respect to 0 mM Amytal (Student's t test for two means).

tent with our earlier estimates from the distribution of K^+ (Dagani and Erecińska, 1987).

Effect of Temperature

All experiments described in this work were performed at 30°C. However, there was no qualitative difference in the results when the temperature was raised to 37°C: the rates of oxygen consumption and lactate production were 50–60% higher and the same degrees of stimulation by veratridine and inhibition by ouabain were observed.

DISCUSSION

The purpose of this work was to evaluate relations between the neuronal Na/K pump activity and cellular ATP synthesis. To accomplish this the effects of three determinants of enzyme activity, $[K^+]_e$, $[Na^+]_i$, and $[ATP]_i$ were characterized in preparations of intact rat brain synaptosomes by determining consequences of alteration in the pump action on respiration, glycolysis, and ouabain-sensitive ^{86}Rb

uptake. This information affords the basis for analysis of three selected aspects of the relationship: (*a*) the distribution of sources of energy for the pump; (*b*) the identity of mechanisms responsible for enhanced energy production during stimulation of ion movements; and (*c*) the role of glycolysis in providing ATP for the Na/K ATPase.

The energy-linked functions of synaptosomes, respiration, glycolysis, and K^+ movements, are affected by the determinants of the Na/K pump action. Potassium stimulates respiration and lactate production with a $K_{0.5}$ of ~1 mM, a value not much different from the K_m for K^+ obtained by measuring the ouabain-sensitive, Na/K-dependent ATPase activity in numerous membrane preparations from nerve (Kimelberg et al., 1978; Grisar et al., 1979; Logan, 1980) and other tissues (e.g. Levinson and Hempling, 1967), as well as in isolated enzyme (Glynn and Karlish, 1975; Schuurmans-Stekhoven and Bonting, 1981). This means that in the CNS in vivo, where extracellular potassium is 3.3–3.5 mM (Hansen, 1985) the Na/K pump is close to saturation with this cation and that rises in its concentration to 10 mM, such as occur during enhanced electrical activity in brain (Nicholson et al., 1978; Somjen, 1979), will contribute only a small fraction to the overall increased work of the pump.

Activities of the ATP-synthesizing pathways are also dependent on $[Na^+]_i$ as demonstrated by large stimulations of O_2 uptake and glycolytic flux by veratridine. Although we did not measure the $K_{0.5}$ for activation of these pathways by sodium, it is generally agreed that the K_m of brain ATPase for Na^+ is 15–20 mM (Kimelberg et al., 1978) similar to that in other tissues (Glynn and Karlish, 1975; Schuurmans-Stekhoven and Bonting, 1981; Sejersted et al., 1988), albeit values as high as 80 mM have been reported (Logan, 1980). The internal $[Na^+]$ in synaptosomes is about 30 mM (Erecińska et al., 1986), that in cerebral neurons 26.7 mM and in glia 19.9 mM (Erecińska and Silver, 1989, 1990), which means that activity of the pump in brain in vivo is predominantly determined by the concentration of $[Na^+]_i$. The observation that with 40 mM KCl increases in the rate of oxygen uptake (Fig. 1) and lactate synthesis (Fig. 4) are smaller than with 6–10 mM KCl is consistent with this suggestion since addition of [KCl] higher than 20 mM has been found to decrease $[Na^+]_i$ (Bührle and Sonnhof, 1983).

Our studies show that cellular energy producing pathways influence operation of the Na/K pump because ATP is a substrate for this enzyme. Isolated Na/K ATPase exhibits two K_m's for ATP, one <1 μM and the other 0.3–0.5 mM (Glynn and Karlish, 1975; Robinson, 1976). The concentration of ATP in "nonstimulated" synaptosomes is about 4 nmol/mg protein, or 1 mM, which indicates that the pump is not fully saturated with the nucleotide. Interestingly, this [ATP] is sufficient to support the activity of the pump at a level that is high enough to balance the leak pathways and maintain constant and close to physiological Na^+ and K^+ gradients. However, even a relatively small decrease in ATP synthesis, such as seen with 1 mM Amytal, reduces activity of the ATPase and leads to a net sodium gain and potassium loss, with consequent membrane depolarization (Dagani and Erecińska, 1987). It may be expected that during intense ion movements caused by veratridine the activity of the Na pump is curtailed, to some extent, by the falling levels of ATP.

The amount of ATP in whole mammalian brain is 2–3 μmol/g wet wt (Siesjö,

1978; Erecińska and Silver, 1989), i.e., 2.5–3.5 mM; thus the operation of the Na pump should be largely independent of the concentration of this nucleotide. However, several studies have shown that in isolated cells from various sources, the rates of ouabain-sensitive K^+ (or ^{86}Rb) transport increase linearly with a rise in [ATP] to 8–10 mM (Ikehara et al., 1984; Soltoff and Mandel, 1984; Tessitore et al., 1986). If this were true for the CNS cells as well, one could surmise that the neuronal Na pump in vivo may not operate at its maximal capacity. Unfortunately synaptosomes lack adenosine kinase activity and we have been unable to raise their adenine nucleotide levels by incubations with adenosine to test this prediction (Erecińska, M., unpublished data).

In addition, the function of the Na/K pump can be influenced by calcium ions which inhibit the enzyme activity (Skou, 1957, Yingst, 1988). This was also observed in our studies in which the ouabain-sensitive increases in respiration and glycolysis caused by addition of veratridine (Tables I and II) were curtailed in the presence of $CaCl_2$.

It is finally worth noting that the transient stimulation of O_2 uptake by high KCl which is dependent on external calcium (Fig. 2 B) but independent of either Ca^{2+} uptake by mitochondria (Fig. 2 C) or activity of the Na/K pump (Fig. 3) is very similar to the observation of Fein and Tsacopoulos (1988) on Limulus ventral photoreceptors. The mechanism(s) of this phenomenon is (are) unknown and its investigation is beyond the scope of the present study.

Having summarized briefly the background information, we shall now discuss selected aspects of the relations between Na/K pump activity and cellular ATP synthesis using as a guideline the three questions posed in the Introduction.

(a) What are the quantitative contributions from glycolysis and oxidative phosphorylation to the energy needed for the operation of the Na/K pump under resting and stimulated conditions? Our results show that synaptosomal Na/K pump is fueled by both oxidative phosphorylation and glycolysis. It can be calculated from the results in Tables I and II and assuming stoichiometric factors of 6 and 1, respectively, for the formation of ATP from the rates of O_2 uptake and lactate production, that in the presence of physiological $[Ca^{2+}]$ the former provides (3.65–2.87) × 6 i.e., 4.68 nmol ATP/min per mg protein and the latter 0.45 nmol/min per mg protein for the operation of the pump (30 min of incubation). Thus glycolysis contributes 0.45 out of 5.13 nmol ATP or 7%. After 5 min of stimulation with veratridine, the Na/K pump utilizes 34.98 nmol ATP/min per mg protein of mitochondrial origin and 1.62 nmol, or 4%, furnished by glycolysis; the proportion of the latter is 8% after 30 min incubation with the alkaloid.

An inescapable conclusion from these calculations is that in synaptosomes, and perhaps in neurons as well (Collins et al., 1969; Borgstrom et al., 1976; Chapman et al., 1977), oxidative phosphorylation provides the predominant proportion of ATP necessary to support the operation of the Na/K pump under both resting and stimulated conditions. This contention is strengthened by three lines of independent evidence. First, the ouabain-sensitive Rb/O_2 ratio is approximately 12, which corresponds to the Rb/ATP ratio of 2, suggesting that almost all the energy required by the pump for K^+ movements is provided in an O_2-consuming process. Secondly, pyruvate in the absence of glucose, is able to maintain unaltered ion gradients and

rates of K^+-transport (Pastuszko et al., 1981; this paper). Thirdly, lactate as a sole energy substrate was found to support normal synaptic function in rat hippocampal slices for hours, without any signs of deterioration (Schurr et al., 1988). Since operation of the Na/K pump is necessary for undisturbed synaptic transmission, the latter observation means, contrary to earlier suggestions (Lipton and Robacker, 1983), that glycolytically produced ATP is not specifically required for the Na/K ATPase to function. The observation by Raffin et al. (1988) that superfusion of rat cerebral cortex with iodoacetate results in K^+ release does not contradict this suggestion because in brain where pyruvate is the main respiratory substrate inhibition of glycolysis also limits the mitochondrial function. Hence it is equally possible that an impairment of mitochondrial ATP synthesis, as shown by an oxidation of cytochrome aa_3 and increase in local oxygen tension (Raffin et al., 1988) was responsible, to a large extent, for a decrease in the Na/K pump activity.

The choice of source of energy for the pump seems to be the prerogative of individual cell types. An examination of the data in the literature shows that some cells, such as renal tubules, utilize predominantly oxidative phosphorylation to support their uphill movements of Na^+ and K^+ (Harris et al., 1980) whereas vascular smooth muscle or cardiac myocytes rely almost exclusively on glycolysis (Paul et al., 1979; Weiss and Hiltbrand, 1985). By contrast, many cells in culture use either pathway with equal efficiency (Van Rossum et al., 1971). Although compartmentation of individual enzymes or pathways may be an answer in some situations (Knull, 1978; Lim et al., 1983), in others a balance between the regulatory factors discussed below will be responsible for the behavior of a particular cell.

(b) *What is the nature of the regulatory mechanisms responsible for stimulation of the energy producing pathways during increased ion pumping?* During short-term changes in cellular function glycolysis is controlled predominantly by the operation of phosphofructokinase (PFK) which is stimulated by ADP, AMP, and P_i and inhibited by PCr and ATP (Passonneau and Lowry, 1962; Ueda, 1979). When energy expenditure is increased, such as occurs during intense ion pumping, the [ADP] and [P_i] rise and [ATP] falls (Table II and e.g., Duffy et al., 1975; Chapman et al., 1977); consequently the activity of PFK increases thus stimulating flux through the glycolytic pathway. This picture may be an oversimplification because in synaptosomes, as well as in brain, there are several homeostatic mechanisms that oppose large changes in these parameters. They include: (a) increased production of ATP from ADP and Pi through oxidative phosphorylation (see below); (b) rephosphorylation of ADP at the expense of PCr via creatine phosphokinase reaction (Meyer et al., 1984); (c) formation of ATP plus AMP from ADP through adenylate kinase (Noda, 1973). All three processes minimize alterations in [ATP] and [ADP]. Thus an interesting conclusion is that during increased ion pumping the activity of PFK may be determined predominantly by its deinhibition by decreasing [PCr] and stimulation by increasing [AMP] and [P_i].

Mechanisms that stimulate mitochondrial oxidative phosphorylation are much more complex. When oxygen is plentiful, the activity of the respiratory chain is controlled by two variables: the phosphorylation state of the cytosolic adenine nucleotides ([ATP]/[ADP][P_i]) or an equivalent (Brand and Murphy, 1987) and the redox state of the intramitochondrial pyridine nucleotides ([NAD^+]/[NADH]) (Erecińska

and Wilson, 1982). At a constant $[ATP]/[ADP][P_i]$, respiratory activity is inversely related to $[NAD^+]_m/[NADH]_m$; when the latter remains unaltered, oxygen uptake rises with a fall in $[ATP]/[ADP][P_i]$. During increased activity of the pump, $[ATP]/[ADP][P_i]$ falls (Table III) which will activate respiration (Table I) and result in oxidation of the intramitochondrial pyridine nucleotides. Such an oxidation has been observed on addition of ADP to isolated mitochondria (Chance and Williams, 1956) and during electrical stimulation of peripheral nerves (Landowne and Ritchie, 1971) and whole brain (Lothman et al., 1975; Lewis and Schuette, 1976). A rise in the $[NAD^+]_m/[NADH]_m$ has two opposing effects on respiration: it decreases the activity of the respiratory chain, because NADH is a substrate for this multienzyme complex, and it stimulates the key mitochondrial dehydrogenases (pyruvate, isocitrate, and 2-oxoglutarate, (Hansford, 1980, for review)) with consequent increase in the production of NADH. Since a decline in $[ATP]/[ADP]$ also activates the same dehydrogenases (Hansford, 1980 for review), transient oxidation of the pyridine nucleotides is likely to be followed by their reduction and an additional increase in oxygen uptake. Our results on pyruvate decarboxylation (Table IV) are consistent with this analysis. The enhanced synthesis of ATP which ensues will raise [ATP] and lower [ADP] and $[P_i]$. This postulated mechanistic explanation of events that follow stimulation of the pump suggests that the primary moving force for the increase in oxidative phosphorylation is the decline in the phosphorylation state of the adenine nucleotides. However, it also indicates that the final level of respiratory activity is equally influenced by the accompanying changes in the $[NAD^+]_m/[NADH]_m$ which are determined, to a large extent, by the regulatory dehydrogenases of the TCA cycle.

However, yet another mechanism independent of those described above, may affect the operation of the respiratory chain: it involves activation of the rate-controlling mitochondrial dehydrogenases by increased levels of $[Ca^{2+}]_i$ (Denton and McCormack, 1985; Hansford, 1985). Such a stimulation raises [NADH] independent of changes in $[ATP]/[ADP][P_i]$ and leads to an increase rate of ATP synthesis. This activation has been shown to occur for pyruvate (Denton et al., 1972), 2-oxoglutarate (McCormack and Denton, 1979), and isocitrate (Denton et al., 1978) dehydrogenases. There is some experimental evidence that Ca^{2+}-dependent rise in the active form of pyruvate dehydrogenase (PDH_a) is responsible for activation of respiration in perfused heart during increases in mechanical activity (Katz et al., 1988) or infusion of epinephrine (McCormack and England, 1983) although other studies correlated the observed stimulation of this enzyme with enhanced myocardial energy utilization (Bünger and Permanetter, 1984). In hippocampal slices, high frequency stimulation increases the fraction of PDH in its active, dephosphorylated form (Browning et al., 1981). In isolated synaptosomes, addition of 24 mM KCl was shown to activate the flux through the PDH complex rapidly but very transiently (Schaffer and Olson, 1980) whereas veratridine administration increased slightly the amount of PDH_a (Hansford and Castro, 1985). It is well documented (see Results) that $[Ca^{2+}]_i$ rises under all conditions cited above. However, our results demonstrate that in the presence of veratridine when cytosolic $[Ca^{2+}]$ increases markedly, neither stimulation of respiration (Fig. 5) nor enhanced decarboxylation of pyruvate (Table IV) are affected by ruthenium red-induced inhibition of calcium entry into mito-

chondria whereas ouabain almost completely eliminates the rise in O_2 uptake (Table I). Thus taking the very small stimulation of respiration by veratridine in the presence of ouabain as a measure of the contribution from direct stimulation of mitochondrial dehydrogenases by calcium it can be concluded that such a mechanism contributes very little to the overall stimulation of oxidative phosphorylation (see also Kauppinen and Nicholls, 1986) and that the main factor controlling mitochondrial ATP production during stimulation of the Na/K pump is the change in [ATP]/[ADP] [P_i] (or an equivalent). This behavior does not appear to be a peculiar characteristic of the synaptosomal system because a similar lack of significant direct activation of respiration by Ca^{2+} at the level of mitochondrial dehydrogenases has been observed recently in isolated cardiac myocytes (Moreno-Sanchez and Hansford, 1988).

(c) *How efficient is glycolysis in supporting the Na/K pump when mitochondrial function is limited?* Our results show that when mitochondrial function is impaired, glycolysis cannot support the operation of the pump because it provides ATP at a level which kinetically limits the enzyme activity. Although this behavior was demonstrated in the synaptosomal preparation, the relatively low [ATP] in brain combined with large requirements for energy for the maintenance of ion gradients might suggest that the same occurs in the CNS in vivo. The plethora of information which shows that limitation in O_2 supply to this organ causes early and massive movements of K^+ and Na^+ with consequent collapse of their gradients (cf. Hansen, 1985 for review) supports this proposition.

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