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# CONTROL OF BRAIN SLICE RESPIRATION BY ($Na^+ + K^+$)-ACTIVATED ADENOSINE TRIPHOSPHATASE AND THE EFFECTS OF ENZYME INHIBITORS

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## SUMMARY

The involvement of membrane ($Na^+ + K^+$)-ATPase ($Mg^{2+}$-dependent, ($Na^+ + K^+$)-activated ATP phosphohydrolase, E.C. 3.6.1.3) in the oxygen consumption of rat brain cortical slices was studied in order to determine whether ($Na^+ + K^+$)-ATPase activity in intact cells can be estimated from oxygen consumption. The stimulation of brain slice respiration with $K^+$ required the simultaneous presence of $Na^+$. Ouabain, a specific inhibitor of ($Na^+ + K^+$)-ATPase, significantly inhibited the ($Na^+ + K^+$)-stimulation of respiration. These observations suggest that the ($Na^+ + K^+$)-stimulation of brain slice respiration is related to ADP production as a result of ($Na^+ + K^+$)-ATPase activity. However, ouabain also inhibited non-$K^+$-stimulated respiration. Additionally, ouabain markedly reduced the stimulation of respiration by 2,4-dinitrophenol in a high ($Na^+ + K^+$)-medium. Thus, ouabain depresses brain slice respiration by reducing the availability of ADP through ($Na^+ + K^+$)-ATPase inhibition and acts additionally by increasing the intracellular $Na^+$ concentration. These studies indicate that the use of ouabain results in an over-estimation of the respiration related to ($Na^+ + K^+$)-ATPase activity. This fraction of the respiration can be estimated more precisely from the difference between slice respiration in high $Na^+$ and $K^+$ media and that in choline, $K^+$ media. Studies were performed with two ($Na^+ + K^+$)-ATPase inhibitors to determine whether administration of these agents to intact rats would produce changes in brain respiration and ($Na^+ + K^+$)-ATPase activity. The intraperitoneal injection of digitoxin in rats caused an inhibition of brain ($Na^+ + K^+$)-ATPase and related respiration, but chlorpromazine failed to alter either ($Na^+ + K^+$)-ATPase activity or related respiration.

## INTRODUCTION

It is widely recognized that the rate of mitochondrial respiration is primarily controlled by the concentration of adenosine diphosphate (ADP) produced by the hydrolysis of adenosine triphosphate (ATP) [1, 2]. While hydrolysis of ATP is accomplished in part by other enzymes [3, 4], a substantial portion may be con-

tributed by the activity of a membrane-bound enzyme $(Na^+ + K^+)$-ATPase ($Mg^{2+}$-dependent, $(Na^+ + K^+)$-activated ATP phosphohydrolase, EC 3.6.1.3) that produces ADP as it pumps $Na^+$ out of and $K^+$ into the cell [5]. Whittam and his co-workers have estimated that the respiration associated with the $(Na^+ + K^+)$-ATPase activity in brain slices is approximately 40 % of the non-stimulated respiration seen in a $Ca^{2+}$-free Krebs Henseleit medium [6–8]. Their estimate depends upon ouabain inhibition of such respiration under conditions in which $K^+$ transport is inhibited.

The present study examines the oxygen consumption of brain slices associated with $(Na^+ + K^+)$-ATPase activity. Data indicate that the fraction of respiration associated with this enzyme activity is substantially smaller than 40 % of the non-stimulated respiration, and that the use of ouabain results in an over-estimation of this portion. A method to more precisely estimate $(Na^+ + K^+)$-dependent portion of the brain slice respiration is proposed. Using this new method, the effects of digitoxin and chlorpromazine on brain $(Na^+ + K^+)$-ATPase activity in intact cells were studied.

## METHODS

Male Sprague-Dawley rats were decapitated and slices were prepared from the cerebral cortex. Slices were immediately weighed and incubated for 20 min at 37 °C in a 100 % oxygen atmosphere in 1.8 ml of an incubation medium containing 128 mM NaCl, 3 mM KCl, 1.23 mM $MgSO_4$, 15 mM sodium phosphate buffer (pH 7.4) and 24 mM glucose. Oxygen uptake was measured manometrically at 37 °C by the method of Umbreit [9] using a Gilson differential respirometer (Gilson Medical Electronics, Inc., Middleton, Wisconsin). The rate of respiration of brain slices was estimated for a 30-min period before the addition of agents. In the first series of experiments, 0.2 ml of either 1.0 M KCl or choline chloride was added at this time to 1.8 ml of the above media containing 0, 1, 10 or 100 $\mu$M ouabain (final concentration of potassium or choline, 100 mM). Respiration was then monitored in duplicate and expressed as $\mu$moles oxygen consumed per gram tissue (wet weight) per 30 min. In some studies, 10 mM pyruvate was used instead of glucose as the substrate. In other experiments, the ionic composition of incubation media was altered as indicated below.

In the studies on intracellular sodium and potassium concentrations, brain cortical slices were prepared and incubated in a $Ca^{2+}$-free Krebs Henseleit solution with various concentrations of ouabain. Tracer amounts (1 $\mu$Ci) of [$^{14}$C]inulin (Amersham/Searle Corporation, Chicago, Ill.) were added to each vessel 15 min before removal of the tissue slices. Tissues were removed after 15, 25 and 35 min incubation at 37 °C. They were blotted, weighed and then homogenized in 2.0 ml of double distilled water. The protein content of each homogenate was assayed by the method of Lowry et al. [10]. Samples were diluted to 1.0 mg protein per ml and digested overnight with an equal volume of nitric acid. Sodium and potassium content was then estimated using an Instrumentation Laboratory Model 143 Flame Photometer according to the method described by Pappius and Elliott [11]. For the estimation of [$^{14}$C]inulin content, a 1.0-ml aliquot of each tissue homogenate was added to 0.2 ml of 50 % (w/v) trichloroacetic acid, mixed well and centrifuged. One milliliter of the acid supernatant from each sample was then counted on a Beckman Model LS-100 Liquid Scintillation Counter, using 15 ml of PCS cocktal (Amersham/Searle Corporation) as the liquid scintillation solution. One hundred microliters of each incubation

medium was also counted for $^{14}C$ radioactivity. The densities of tissue slices were estimated by their sedimentation in various concentrations of sucrose solution, and total tissue slice volumes were calculated from densities and tissue weights. The extracellular volume of each slice was calculated from the [$^{14}C$]inulin data and this value was subtracted from total slice volume to give the intracellular volume. The intracellular sodium and potassium concentrations in mequiv./liter were calculated for each brain slice from extracellular sodium and potassium concentrations; total, extracellular and intracellular volumes, and total sodium and potassium concentrations, as follows:

$$(V_t)\,(C_t) = (V_i)\,(C_i) + (V_0)\,(C_0)$$

where $V_t$ = total volume, $V_i$ = intracellular volume, $V_0$ = extracellular volume, $C_t$ = total sodium or potassium concentration, $C_i$ = intracellular sodium or potassium concentration and $C_0$ = extracellular sodium or potassium concentration.

In an experiment designed to study the effects of $Na^+$ and $K^+$ on brain cortical homogenate respiration, the method described by Potter [12] was used with a slight modification. Using a Potter-Elvehjem homogenizer and Teflon pestle driven at 800 rev./min, 20 % homogenates of rat brain cortical slices were prepared in 0.32 M sucrose solution. All solutions and homogenates were kept on ice until incubation was begun. The homogenate (0.25 ml) was added to an incubation medium (1.75 ml) resulting in final concentrations of 2.0 mM Tris-ATP, 3.0 mM $MgCl_2$, 10 mM potassium phosphate buffer (pH 7.2), 10 mM glucose, 0.05 mM dipotassium EDTA, 0.2 mM NAD, 40 mM nicotinamide and 40 $\mu$g hexokinase enzyme per ml (activity 18.5 units/mg protein, Sigma Chemical Company, St. Louis, Mo.). Final sodium and potassium concentrations were varied from 0 to 120 mM using choline chloride as an osmotic substitute and maintaining the cation concentration at 120 mM. The time from decapitation of the rat to the start of incubation was approximately 17 min while the total time elapsed before taking the first reading was approximately 30 min. The homogenate was incubated at 30 °C in a 100 % oxygen atmosphere. The protein concentration in each homogenate was assayed using the biuret method as described by Gornall et al. [13]. Data were expressed in terms of $\mu$mol oxygen consumed per milligram protein during the initial 15-min period.

In experiments in which effects of digitoxin or chlorpromazine administration were studied, male Sprague-Dawley rats weighing 200–250 g were injected intraperitoneally with digitoxin dissolved in ethyl alcohol or chlorpromazine hydrochloride dissolved in 0.9 % NaCl solution. Control rats received comparable volumes of the vehicle. Rats were sacrificed 30 min after injection, and cortical slices and whole brain homogenates were prepared. In brain slice experiments, cortical slices were incubated at 37 °C for 1.0 h in 2.0 ml of either a medium containing (A) 128 mM NaCl, 103 mM KCl, 1.23 mM $MgSO_4$, 15 mM sodium-phosphate buffer (pH 7.4) and 24 mM glucose, or (B) a similar medium in which 128 mM choline chloride and 15 mM Tris-phosphate buffer were substituted for NaCl and sodium-phosphate buffer, respectively, to yield a $Na^+$-free incubation medium. Respiration rates in both ($Na^+ + K^+$)-medium and choline, $K^+$-medium were calculated in $\mu$moles of oxygen consumed per gram tissue (wet weight) per 30 min. Differences between rates of oxygen consumption in the ($Na^+ + K^+$)-media and those in choline, $K^+$-media were calculated. In ATPase activity experiments, 5 % whole brain homogenates were prepared at 0° as described

above using a medium containing 0.25 M sucrose, 5.0 mM histidine and 1.0 mM Tris-EDTA (pH 7.0). $(Na^+ + K^+)$-ATPase and $Mg^{2+}$-ATPase activities of the homogenate were estimated immediately from the amount of $P_i$ liberated from ATP during a 10-min incubation at 37 °C as described previously [14]. Protein was estimated by the method of Lowry et al. [10].

Unless otherwise stated, statistical analysis in all experiments was performed by a completely randomized design analysis of variance using the Student-Newman-Keuls test to determine significant differences between means [15]. Criterion for significance was a $P$ value of less than 0.05.

## RESULTS

### *In vitro slice respiration studies*

In the first series of experiments, brain slices were incubated for a 30-min period at 37 °C in a $Ca^{2+}$-free Krebs-Henseleit solution (pH 7.4) which contained 143 mM $Na^+$, 3 mM $K^+$ and 24 mM glucose with either 0, 1, 10 or 100 $\mu$M ouabain. Following the first 30-min incubation, either KCl or choline chloride (final concentration after the addition, 100 mM) was added from the flask sidearm to the incubation medium and the slice respiration was monitored for an additional 30-min period. Fig. 1A shows the data obtained during the second 30-min period. The hatched bars represent the respiration after the choline addition and the entire bars, total respiration after the addition of 100 mM KCl. The open bars thus represent the $K^+$-stimulated oxygen consumption. It may be observed that $K^+$ caused an approximately 27 % increase in respiration under these conditions in the absence of ouabain. The

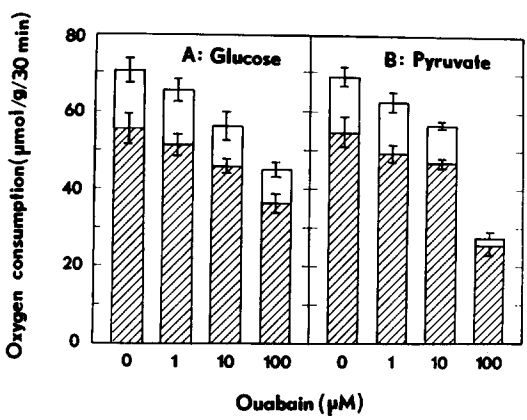


Fig. 1. Effects of potassium and ouabain on brain slice respiration in $Ca^{2+}$-free media with glucose or pyruvate substrate. Oxygen consumption of rat brain cortical slices was assayed in a medium containing 128 mM NaCl, 3 mM KCl, 1.23 mM $MgSO_4$, 15 mM sodium phosphate buffer (pH 7.4) with either 24 mM glucose (panel A) or 10 mM pyruvate (panel B). After a 30-min incubation at 37 °C in the presence or absence of ouabain, either KCl or choline chloride (final concentrations, 100 mM) was added. Oxygen consumption during a 30-min period following the addition of KCl (total bars) or choline chloride (shaded bars) was expressed as $\mu$mol/g tissue (wet weight). Mean of four experiments. Vertical lines indicate S.E.M. Choline and total respiration were significantly different from control in the presence of 10 and 100 $\mu$M ouabain with glucose and 100 $\mu$M ouabain with pyruvate ($P < 0.05$).

addition of choline had no effect on brain slice respiration: the first and the second 30-min respiration, i.e., the respiration before and after the addition of choline, being 53.7 and 55.0 $\mu$mol oxygen consumed per gram tissue (wet weight), respectively.

It may also be observed in Fig. 1A that the effect of $K^+$ addition on the respiration was significantly reduced at the two higher concentrations (10 and 100 $\mu$M) of ouabain. Furthermore, the non-stimulated portion of the brain slice respiration (the hatched bars) was reduced by 34 % following incubation with 100 $\mu$M ouabain. This latter finding is consistent with that reported previously by Whittam and his co-workers [6–8].

Thus, it would appear that both $K^+$-stimulated respiration (with 100 mM $K^+$) and non-stimulated respiration (with 100 mM choline) could be inhibited by ouabain in a concentration-related fashion, although the $K^+$-stimulated portion of the respiration was more sensitive to ouabain than was the non-stimulated portion.

In a parallel series of experiments, 10 mM pyruvate was utilized as the substrate instead of glucose (Fig. 1B), in order to rule out the possibility that the effects of ouabain on slice respiration observed with glucose was substrate specific. The data, obtained with pyruvate, are similar to those seen with glucose. Control respiration during the first 30 min period was 60.5 $\mu$mol of oxygen consumed per g tissue (wet weight) in 30 min and the addition of 100 mM KCl stimulated the respiration by 26 %. A concentration-related inhibition of both the $K^+$-stimulated and non-stimulated respiration was observed with an almost complete abolition of the $K^+$-stimulated respiration with 100 $\mu$M ouabain. Non-stimulated respiration during the second half hour period was reduced by approximately 50 % with this concentration of ouabain.

In attempts to relate ouabain's effect on respiration to tissue $(Na^+ + K^+)$-

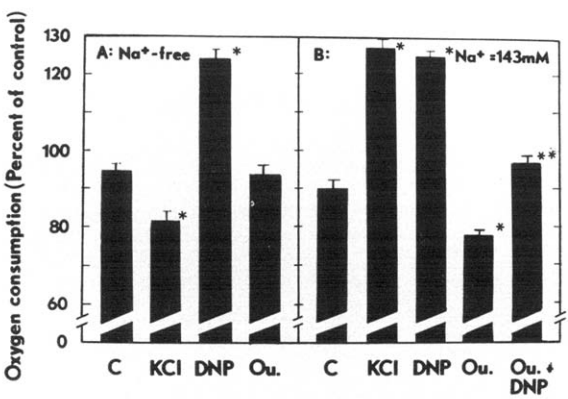


Fig. 2. Effects of potassium, 2,4-dinitrophenol and ouabain on brain slice respiration in the absence and presence of sodium. Oxygen consumption of rat brain cortical slices was assayed in a medium in which NaCl and sodium phosphate buffer were replaced by choline chloride and Tris · HCl buffer, respectively (panel A) or in a medium described in legend to Fig. 1 (panel B), using glucose as the substrate. Following a 30-min incubation at 37 °C, either KCl (final concentration, 100 mM), 2,4-dinitrophenol (final concentration, 50 $\mu$M) or ouabain (final concentration, 100 $\mu$M) or the combination of 2,4-dinitrophenol and ouabain was added. Oxygen consumption during a 30-min period following the addition was expressed as a percent of that prior to addition. Mean of ten experiments. Vertical lines indicate S.E.M. *Significantly different from control ($P < 0.05$). **Significantly different from the value observed following the addition of 2,4-dinitrophenol alone ($P < 0.05$).

ATPase activity, studies on oxygen consumption in a $Na^+$-free medium were undertaken. In these experiments, choline chloride now replaced NaCl, and Tris-phosphate was used instead of sodium phosphate buffer. Glucose was the substrate. The mean value for the first half hour oxygen consumption in a $Na^+$-free (choline) medium was 44.7 $\mu$mol per g tissue (wet weight) in 30 min. This value is significantly lower than the value obtained in the medium containing high sodium, such as the $Ca^{2+}$-free Krebs-Henseleit medium. Data for the second 30 min period following the addition of choline, ouabain, KCl or 2,4-dinitrophenol are shown in Fig. 2A. It may be observed that choline addition in the $Na^+$-free medium reduced the respiration (compared to the pre-addition period) during the second half hour by 5 %, while KCl, in the absence of $Na^+$, produced a greater (19 %) reduction in the respiration rather than a stimulation of oxygen consumption. This would indicate that $Na^+$ is required for $K^+$ to stimulate brain slice respiration (Figs. 2A and B). The addition of 2,4-dinitrophenol to brain slices in a $Na^+$-free medium resulted in a significant increase in respiration, attesting to the viability of the tissue and also indicating that $Na^+$ has no function once respiratory control is removed. Ouabain (100 $\mu$M) has a minimal effect on respiration in the absence of $Na^+$.

Since $K^+$ failed to stimulate brain slice respiration in a $Na^+$-free medium, an attempt was made to demonstrate the necessity of the simultaneous presence of $Na^+$ and $K^+$ for the stimulation of brain slice respiration. In order to do these studies, a medium in which sodium was replaced by potassium was utilized. In some flasks, ouabain was added prior to the first 30-min incubation period. The data in Fig. 3 show the results obtained during a 20-min period following the addition of NaCl (final concentration, 128 mM), or in control flasks, the addition of choline. The mean

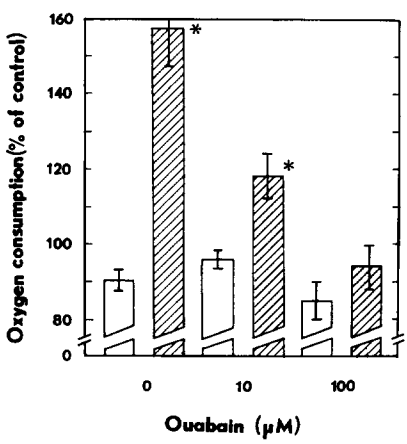


Fig. 3. Effects of ouabain on sodium-stimulation of brain slice respiration in a high $K^+$ medium. Oxygen consumption of rat brain cortical slices was assayed in a medium containing 103 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer (pH 7.4) and 24 mM glucose. After a 30-min incubation at 37 °C in the presence or absence of ouabain, either NaCl (hatched bars) or choline chloride (open bars) (final concentrations, 128 mM) was added. Oxygen consumption during a 30-min period following the addition was expressed as a percent of that prior to the addition. Mean of ten experiments. Vertical lines indicate S.E.M. *Significantly different from corresponding choline controls ($P < 0.05$).

control oxygen consumption of brain slices in the KCl medium prior to the addition was 41.6 μmol per g tissue (wet weight) in 30 min. Respiration in the KCl medium was reduced by approximately 10 % during the second 30 min of incubation following the addition of choline chloride (Fig. 3). As may be seen, the addition of $Na^+$ now produced a large increase in respiration compared to the corresponding addition of choline chloride (bar 1 vs. 2, Fig. 3). Furthermore, both concentrations of ouabain (10 and 100 μM) significantly reduced this stimulation. Ouabain, however, even at the higher concentration failed to significantly inhibit the brain slice respiration when no $Na^+$ was present.

In the next series of experiments, the concentration-relationship of $Na^+$-induced stimulation of brain slice respiration was explored. In these studies, the pre-addition medium contained 103 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer (pH 7.4) and 24 mM glucose. The mean oxygen consumption during the first 30 min period in this medium was 40.5 μmol per g tissue (wet weight) in 30 min. Various combinations of choline and $Na^+$ were added to give a final concentration of 100 mM and the respiration was monitored for several 30-min periods. The data from these studies are shown in Fig. 4. Additions of $Na^+$ to give a final concentration ranging from 10–100 mM significantly stimulated brain slice respiration (21–71 %) in a concentration dependent manner. This $Na^+$-induced stimulation was sustained for at least 2 h at all $Na^+$ concentrations.

Thus, it appears that the $K^+$-induced stimulation is in fact the result of the simultaneous presence of $Na^+$ and $K^+$ similar to the stimulation of $(Na^+ + K^+)$-ATPase activity by these cations. However, that the inhibitory action of ouabain on brain slice respiration is not solely dependent on a decreased availability of ADP to participate in the phosphorylation-coupled respiration via $(Na^+ + K^+)$-ATPase inhibition by this agent can be inferred from the earlier observations presented in Fig.

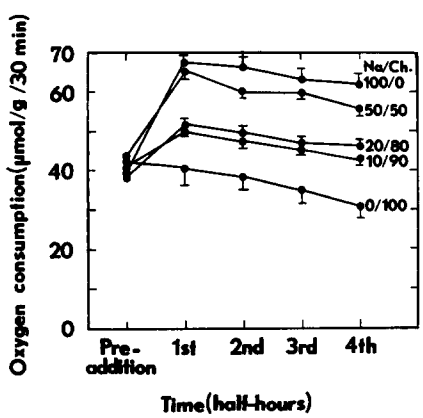


Fig. 4. Time and concentration effects of $Na^+$-stimulation of brain slice respiration in a high $K^+$ medium. Oxygen consumption of rat brain cortical slices was assayed in a medium containing 103 mM KCl, 1.23 mM $MgSO_4$, 15 mM Tris-phosphate buffer (pH 7.4) and 24 mM glucose. After a 30-min incubation at 37 °C, NaCl was added yielding final $Na^+$ concentrations of 0, 10, 20, 50 or 100 mM. Choline chloride was used as an osmotic substitute so that osmolarity following the addition was increased equally in all vessels. Oxygen consumption was monitored for four 30-min periods following the addition. Mean of four experiments. Vertical lines indicate S.E.M.

2B. In these studies, slice oxygen consumption was measured in a $Ca^{2+}$-free Krebs-Henseleit medium with glucose substrate. Following a 30-min control incubation, either 2,4-dinitrophenol, ouabain or a combination of 2,4-dinitrophenol and ouabain was added to the flasks and the respiration was monitored for an additional 30-min period. The same amount of a $Ca^{2+}$-free Krebs-Henseleit solution was added to control flasks. Preaddition control oxygen consumption was 58.5 $\mu$mol per g tissue (wet weight) in 30 min. Control respiration during the second half-hour period was approximately 90 % of the pre-addition level (Fig. 2B). The addition of 2,4-dinitrophenol now significantly increased respiration (approximately 33 %) compared to the corresponding control value, whereas 100 $\mu$M ouabain caused a significant reduction in respiration (approximately 22 %). In the presence of ouabain, 2,4-dinitrophenol failed to stimulate the respiration to a level observed with 2,4-dinitrophenol in the absence of ouabain. Since this concentration of 2,4-dinitrophenol has been shown to induce a maximal uncoupling of oxidative phosphorylation (unpublished observations), it might be anticipated that the stimulant action of 2,4-dinitrophenol would be unaffected by ouabain if the reduced availability of ADP was the only mechanism for the inhibition of brain slice respiration by this agent. That this result was not observed (Fig. 2B) led us to explore alternative possibilities.

Therefore, the effect of ouabain on intracellular sodium and potassium concentrations was studied (Fig. 5). Tissue slices were prepared and incubated in a similar manner as in the oxygen consumption studies using a medium containing either 0, 10 or 100 $\mu$M ouabain for 15, 25 or 35 min, removed from the incubation media and assayed for intracellular sodium and potassium concentrations. Mean

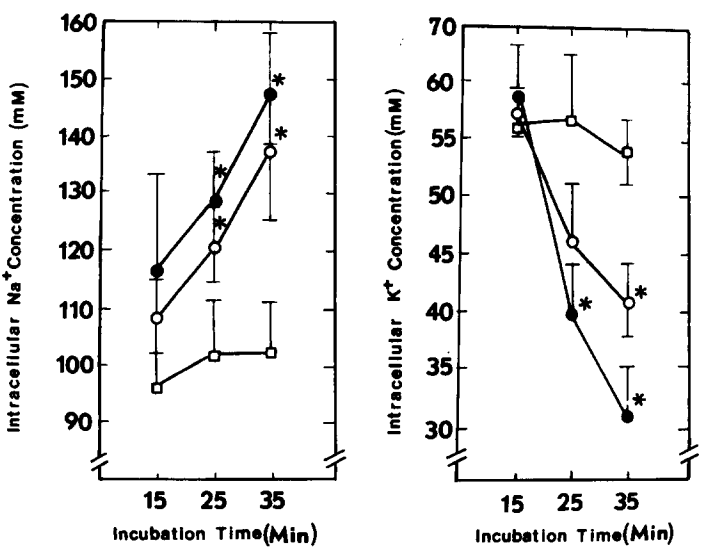


Fig. 5. Effects of ouabain on intracellular sodium and potassium concentrations in rat brain slices. Rat brain cortical slices were incubated at 37 °C in a $Ca^{2+}$-free Krebs medium containing no ouabain (□), 10 $\mu$M ouabain (○) or 100 $\mu$M ouabain (●). After an incubation for an indicated time period, intracellular sodium and potassium concentrations were determined. Mean of four separate experiments. Vertical lines indicate S.E.M. *Significantly different from corresponding values in the absence of ouabain ($P < 0.05$).

control intracellular sodium and potassium concentrations were approximately 100 mM and 55 mM, respectively, after incubation times ranging from 15 to 35 min. Incubation in both concentrations of ouabain resulted in a significant rise in the intracellular sodium concentration (to 147 mM after 35 min in the presence of 100 $\mu$M ouabain) with a simultaneous fall in the intracellular potassium concentration (to 31 mM under the same conditions). All changes appeared to be time and ouabain-concentration dependent. These data on intracellular ions are consistent with the known inhibitory effect of the cardiac glycosides on monovalent cation pump activity.

Based on the observations seen earlier on brain slice respiration and on the above intracellular cation studies, it seemed possible that either increases in intracellular sodium or decreases in intracellular potassium might play an important role in the ouabain inhibition of brain slice respiration, in addition to ouabain's inhibitory effect on the availability of ADP.

To further assess this possibility, the effects of either $Na^+$ increase or $K^+$ decrease on oxygen consumption was studied with brain homogenates respiring at high rates in the presence of excess ADP and Pi. Sodium and potassium concentrations were varied and glucose, NAD, nicotinamide and hexokinase were added to the standard incubation medium (Fig. 6). Reducing the $K^+$ concentration from 145 to 25 mM had no significant effect on brain homogenate oxygen consumption. However, increasing the $Na^+$ concentration from 0 to 120 mM produced a concentration-dependent fall in respiration, an inhibition of approximately 27 % being observed at the highest $Na^+$ concentration. From these studies, it would appear that the ouabain inhibition of brain slice respiration may result from an indirect effect of ouabain to

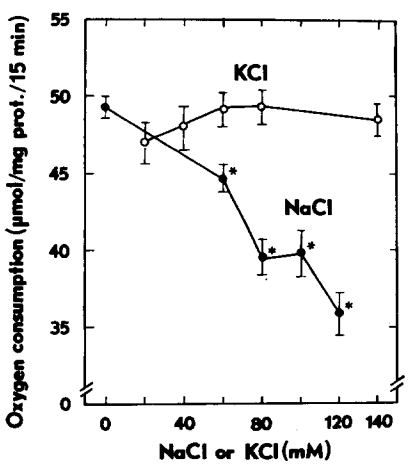


Fig. 6. Effects of sodium and potassium on oxygen consumption of brain homogenates. Rat brain cortical slices were homogenized in 0.32 M sucrose at 0 °C. Homogenate was then incubated at 30 °C in a medium containing 2 mM Tris-ADP, 3 mM $MgCl_2$, 0.2 mM NAD, 40 mM nicotinamide, 40 $\mu$g/ml hexokinase enzyme (activity, 18.5 units/mg), 10 mM glucose, 0.05 mM dipotassium EDTA and 10 mM potassium phosphate buffer (pH 7.2) and various concentrations of NaCl and KCl. NaCl (●) or KCl (○) concentration was varied independently while maintaining the concentration of the other salt (KCl or NaCl, respectively) at 120 mM. Choline chloride was used as an osmotic substitute. Mean of five experiments. Vertical lines indicate S.E.M. *Significantly different from the value in the absence of NaCl ($P < 0.05$).

increase the intracellular $Na^+$ concentration in addition to the direct effect of ouabain on $(Na^+ + K^+)$-ATPase which would result in an inhibition of ADP generation. If this is the case, then the use of cardiac glycosides, such as ouabain, proposed by Whittam and his co-workers [6–8] would overestimate the $(Na^+ + K^+)$-ATPase associated respiration of the brain slice. A more precise estimation of the brain slice respiration associated with this enzyme activity may be obtained from the difference between brain slice respiration in a high $K^+$, high $Na^+$ medium and that in a high $K^+$, $Na^+$-free medium (for a detailed discussion, see below).

*Effects of digitoxin and chlorpromazine administration in vivo on brain slice respiration in vitro*

Estimation of brain slice respiration associated with $(Na^+ + K^+)$-ATPase activity makes it possible to study the effects of agents on this enzyme activity in intact brain cells. Thus, studies were performed in rats to determine whether a systemic administration of cardiac glycosides results in changes in brain $(Na^+ + K^+)$-ATPase activity and respiration. Digitoxin was used instead of ouabain in these studies because it penetrates into the central nervous system more easily than ouabain. In some animals, various concentrations of digitoxin dissolved in alcohol were administered intraperitoneally while other control animals received the solvent. Oxygen consumption of brain cortical slices obtained from control animals was 75.1 and 45.6 $\mu$mol/g tissue (wet weight) in 30 min in high $(Na^+ + K^+)$-media and $Na^+$-

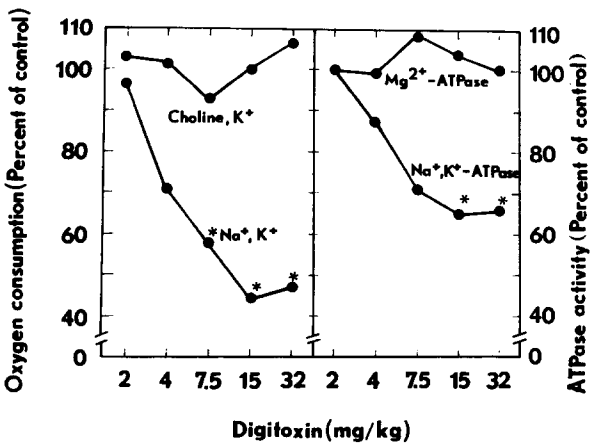


Fig. 7. Effects of digitoxin injection in rats on brain slice respiration and ATPase activities assayed in vitro. Male Sprague-Dawley rats were intraperitoneally injected with digitoxin and sacrificed 30 min later. Oxygen consumption of brain cortical slices was assayed at 37 °C in either a high $(Na^+ + K^+)$ medium containing 128 mM NaCl, 103 mM KCl, 1.23 mM $MgSO_4$, 15 mM sodium phosphate buffer (pH 7.4) and 24 mM glucose or a $Na^+$-free medium in which NaCl and sodium phosphate buffer were substituted for by choline chloride and Tris-phosphate buffer, respectively (left panel). ATPase activities of brain cortical homogenates was assayed at 37 °C in either a medium containing 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris · HCl buffer (pH 7.5) or a medium containing 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris · HCl buffer (pH 7.5) (right panel). Results were expressed as percent of corresponding control value observed with paired animals injected with the vehicle for digitoxin (alcohol). Mean of four experiments. Vertical lines indicate S.E.M. *Significantly inhibited ($P < 0.05$).

free media, respectively. When brain slices were prepared from animals treated intraperitoneally with digitoxin in doses of 1, 2 or 4 mg/kg, there were no significant effects of digitoxin on brain slice respiration either in the medium containing $Na^+$ and $K^+$ (total respiration) or choline and $K^+$ (non-stimulated respiration) (Fig. 7). However, with larger doses of digitoxin (7.5, 15 and 30 mg/kg, intraperitoneally), there was an inhibition of respiration in a medium containing $Na^+$ and $K^+$ but no depression of respiration in a medium containing choline and $K^+$.

As an indicator of the role of $(Na^+ + K^+)$-ATPase in brain slice respiration studies, the activity of this enzyme following the intraperitoneal injection of digitoxin was assayed in brain homogenates (Fig. 7). In these studies, $(Na^+ + K^+)$-ATPase and $Mg^{2+}$-ATPase activities of control rat brain homogenates were 7.72 and 16.4 $\mu$mol $P_i$ liberated per mg protein per h, respectively. All values shown are calculated as percent of the control activity of paired experiments. At the highest digitoxin dose, a 35 % inhibition of $(Na^+ + K^+)$-ATPase activity was observed and the inhibition was dose-dependent. There were no significant changes in $Mg^{2+}$-ATPase activity at any digitoxin dose. Thus, intraperitoneal injection of digitoxin causes an inhibition of rat brain $(Na^+ + K^+)$-ATPase activity in intact cells as well as in homogenates.

A number of investigators have reported that chlorpromazine inhibits $(Na^+ + K^+)$-ATPase when this agent was added to an incubation mixture containing homogenates or partially purified $(Na^+ + K^+)$-ATPase preparations [16–20]. Therefore, it was of interest to determine whether the administration of chlorpromazine in rats causes a brain $(Na^+ + K^+)$-ATPase inhibition with a subsequent reduction in the brain slice respiration associated with this enzyme activity. When brain slices were prepared from animals treated intraperitoneally with chlorpromazine in a dose of 30 mg/kg, there were no significant effects of this drug on the slice respiration either in a medium containing $Na^+$ and $K^+$ or in a medium containing choline and $K^+$ (Fig. 8). This dose of chlorpromazine also failed to affect the brain $(Na^+ + K^+)$-ATPase activity when the enzyme activity was assayed with brain homogenates prepared from animals treated with the drug. Since this dose of chlorpromazine produces catatonia

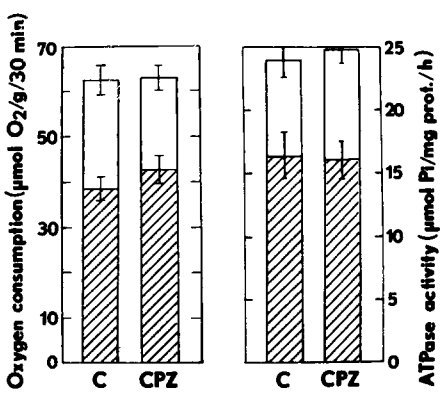


Fig. 8. Effects of chlorpromazine injection to rats on brain slice respiration and ATPase activities assayed in vitro. See legend to Fig. 7. Chlorpromazine hydrochloride (30 mg/kg, intraperitoneally) in physiological saline solution was injected 30 min prior to sacrifice.

after administration, further studies with higher doses of chlorpromazine were not attempted.

## DISCUSSION

An attempt has been made to determine that fraction of brain slice respiration associated with the energy-requiring transport of cations as proposed by Whittam and his co-workers [6–8]. Specifically in question is the suitability of using ouabain-induced inhibition of the respiration as a measure to estimate that fraction of brain slice respiration dependent on active sodium and potassium transport. The present data are consistent with the premise that $(Na^+ + K^+)$-ATPase is responsible for a portion of brain slice respiration in a high $Na^+$, high $K^+$ medium. However, the present study also indicates that the use of ouabain is not suitable to quantify $(Na^+ + K^+)$-ATPase-related respiration.

The dose-dependent inhibition of rat brain slice respiration by ouabain in a modified $Ca^{2+}$-free Krebs-Henseleit medium confirms similar observations in cortical slices from rabbits [6–8]. The finding by previous investigators that ouabain inhibits non-stimulated brain slice respiration was extended further by the present findings in that ouabain inhibits both non-stimulated and $K^+$-stimulated slice respiration. $K^+$-stimulated respiration was more sensitive to ouabain than non-stimulated respiration.

The present experiments also demonstrate that the addition of 100 mM KCl to slices in a $Na^+$-free medium produces a depression of respiration, rather than a stimulation, indicating that there is a specific $Na^+$ requirement for the $K^+$-stimulation of the brain slice respiration. These findings further suggest that the $K^+$-stimulation of the brain slice respiration is not simply due to a $K^+$-induced depolarization of the cell membrane or a $K^+$-induced stimulation of tissue metabolism, but is indeed a $Na^+$ and $K^+$ dependent stimulation of respiration, analogous to the $Na^+$ and $K^+$ dependent stimulation of ATP hydrolysis by $(Na^+ + K^+)$-ATPase.

In electrically depolarized guinea pig cortical slices, increases in sodium concentration in the media are associated with increases in the intracellular sodium concentration and in slice respiration [21]. Furthermore, the present finding that ouabain blocks the $Na^+$-stimulation of brain slice respiration in high $K^+$ media indicates that the $Na^+$ plus $K^+$ stimulated portion of the respiration is specifically associated with $(Na^+ + K^+)$-ATPase enzyme activity.

The use of the $K^+$-stimulated portion of brain slice respiration to determine the ion transport component of respiration, however, would probably result in an underestimation of such a portion of the respiration, since $(Na^+ + K^+)$-ATPase may be partially activated under the conditions in which non-stimulated respiration is observed. The use of ouabain to inhibit $(Na^+ + K^+)$-ATPase related respiration, on the other hand, results in an over-estimation of this fraction of slice respiration, although ouabain has been shown to be a specific inhibitor of $(Na^+ + K^+)$-ATPase in semi-purified enzyme preparations and a specific inhibitor of the active $Na^+$ and $K^+$ transport in various tissues (see ref. 22). If ouabain's mechanism for the inhibition of brain slice respiration is exclusively dependent on the inhibition of ADP production due to $(Na^+ + K^+)$-ATPase inhibition, it would be expected that 2,4-dinitrophenol would cause a similar stimulation of respiration either in the presence or absence of ouabain. Since the level of respiration observed in the presence of 50 $\mu$M 2,4-dinitro-

phenol, a concentration that completely uncouples oxidative phosphorylation [2], was significantly lower in the presence of ouabain than in its absence, there must be another factor(s) responsible for the ouabain-induced reduction in respiration in addition to the inhibition of ADP generation.

Terner et al. [23] showed that brain cortical slices lose potassium when respiration is inhibited with respiratory poisons, while Swanson [24] demonstrated that ouabain induced a potassium loss and a simultaneous sodium gain in rabbit brain slices incubated in a $Ca^{2+}$-free Krebs-Henseleit medium. The present findings confirm these observations and additionally indicate that the ouabain-induced increase in intracellular sodium concentration is of sufficient magnitude to affect brain tissue respiration. Thus, the magnitude of the brain slice respiration inhibited by ouabain corresponds to the inhibition of brain slice respiration related to a reduced ADP availability due to $(Na^+ + K^+)$-ATPase inhibition plus the $Na^+$-induced inhibition of a portion of the brain slice respiration unrelated to $(Na^+ + K^+)$-ATPase activity. Swanson [24] has also suggested that ouabain-induced inhibition in $Ca^{2+}$-deficient media is not a good indicator of the portion of the cell's metabolism used for active transport, based upon his observation that ouabain significantly inhibits the incorporation of $^{32}P$-labelled phosphate into creatine phosphate.

A method for a more accurate estimation of the cell respiration due to ADP generation by $(Na^+ + K^+)$-ATPase system is shown in Fig. 9. In the presence of high $Na^+$ and $K^+$, brain slice respiration is approximately 30 % higher than that in a high $Na^+$, low $K^+$ medium such as Krebs-Henseleit solution. In a $Na^+$-free medium containing high concentrations of choline and $K^+$, the level of brain slice respiration is approximately 15–20 % lower than that in a high $Na^+$, low $K^+$ medium. Since the intracellular sodium concentration rapidly approaches zero when brain slices were incubated in a $Na^+$-free medium containing high concentrations of choline and $K^+$ (data not shown), ATP hydrolysis by $(Na^+ + K^+)$-ATPase should be negligible under these conditions. Thus, the difference in brain slice respiration observed in a high $Na^+$ and $K^+$ media and that observed in a $Na^+$-free media containing high concentra-

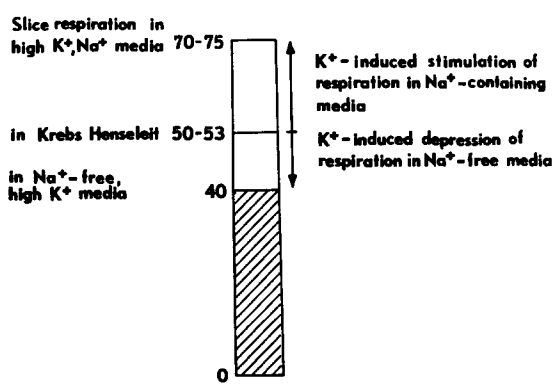


Fig. 9. Components of brain slice respiration associated with $(Na^+ + K^+)$-ATPase. Brain slice respiration independent of $(Na^+ + K^+)$-ATPase activity is shown by the shaded portion of the bar and that dependent on $(Na^+ + K^+)$-ATPase is shown by the open portion of the bar. Numbers are oxygen consumption in $\mu$mol per g tissue (wet weight) per 30 min. For approximate conversion to $Q_{O_2}$, multiply by 2/5.

tions of choline and $K^+$ appears to yield a more accurate measure of the brain slice respiration associated with stimulated, active cation transport, than that obtained using ouabain-induced inhibition of the respiration in the presence of high $Na^+$ and $K^+$. While it would appear superficially that the contribution of $(Na^+ + K^+)$-ATPase activity to overall brain respiration in the present study (which is calculated to be about 45 %) is not different from the estimation of Whittam [7] who concluded that the active transport of sodium and potassium accounts for 40 % of the brain's respiration, these observations are derived from widely disparate data. In Whittam's study, the incubation medium contained only 5 mM KCl, a concentration which is clearly inadequate to mimic functioning brain cells which are continuously firing. Using Whittam's experimental conditions [7] we observed that the portion of brain slice respiration related to $(Na^+ + K^+)$-ATPase activity constituted only 10–15 % of the total respiration. When 100 mM KCl was substituted for 5 mM KCl, we were able to achieve a total slice respiration that was almost twice that of Whittam's, with 50 % attributable to $(Na^+ + K^+)$-ATPase activity (Fig. 9).

Intraperitoneal (systemic) administration of digitoxin to the rat caused an inhibition of the brain $(Na^+ + K^+)$-ATPase activity in intact cells as well as in homogenates. This finding is in contrast to our previous observation in the dog that the intravenous infusion of ouabain failed to alter the brain $(Na^+ + K^+)$-ATPase activity [25]. The differences in results may be partly due to the differences in cardiac glycoside employed. Digitoxin being one of the more lipid-soluble of the cardiac glycosides, penetrates more readily across lipid cell membranes and into the brain. The major reason for the difference, however, is the animal species employed. Rat brain $(Na^+ + K^+)$-ATPase is inhibited by low concentrations of cardiac glycoside whereas rat cardiac $(Na^+ + K^+)$-ATPase is relatively insensitive to these agents [26]. This difference in sensitivity makes it possible to administer relatively large doses of cardiac glycoside to affect the brain enzyme in the rat without causing the death of the animal due to cardiac toxicity. This is not the case with dog since brain and cardiac $(Na^+ + K^+)$-ATPases have a similar relatively high sensitivity to cardiac glycosides. Therefore, the present finding does not indicate that brain $(Na^+ + K^+)$-ATPase may be inhibited following the administration of cardiac glycosides in digitalis sensitive species, whereas such may be the case in animal species in which brain enzyme is markedly more sensitive to digitalis than is cardiac enzyme.

The intraperitoneal injection of digitoxin in the rat resulted in inhibition of brain slice respiration at lower doses than were required for significant effects on $(Na^+ + K^+)$-ATPase activity. The magnitude of digitoxin-induced inhibition of $(Na^+ + K^+)$-stimulated respiration was also greater than that of digitoxin-induced inhibition of $(Na^+ + K^+)$-ATPase activity. The greater inhibition of slice respiration in the presence of high concentrations of $Na^+$ and $K^+$ also suggests that inhibition of respiration may involve both a decrease in ADP generation and an inhibitory effect of intracellular sodium on respiration secondary to the inhibition of the sodium pump activity, as is the case with studies with ouabain in vitro. The lack of effect of digitoxin on slice respiration in the absence of $Na^+$ would be consistent with studies in vitro in which the cardiac glycosides had no effect on respiration in a $Na^+$-free medium.

Unlike digitoxin, chlorpromazine failed to affect brain $(Na^+ + K^+)$-ATPase activity in intact cells and in homogenates when this agent was administered intraperitoneally. We have shown previously that the inhibition of $(Na^+ + K^+)$-ATPase by

chlorpromazine involves the formation of free radical metabolites [14, 27, 28]. Thus, it appears that chlorpromazine free radical is not formed in the brain in amounts necessary to affect $(Na^+ + K^+)$-ATPase activity of cortical slices or whole brain homogenates following the intraperitoneal injection of chlorpromazine. This, however, does not rule out the possibility that free radical formation occurs at a certain specific anatomical site within the central nervous system and therefore specific analyses of various areas of the brain may be needed to clarify this discrepancy.

In summary, the present study demonstrates a method to accurately estimate that fraction of brain slice respiration associated with $(Na^+ + K^+)$-ATPase activity. Using this method, it is possible to study the effects of various agents on brain $(Na^+ + K^+)$-ATPase activity in intact cells following drug administration to the intact animal.

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