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THE EFFECT OF OUABAIN, MERALLURIDE AND ETHACRYNIC ACID ON RESPIRATION AND GLYCOLYSIS IN KIDNEY SLICES

VERNON D. JONES* and ERWIN J. LANDON

Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tenn., U.S.A

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Abstract—The effects of ouabain, meralluride, and ethacrynic acid on glycolysis and respiration of rat and rabbit kidney slices have been investigated. When slices were incubated under conditions favorable to glycolysis, ouabain and meralluride inhibited lactate formation with glucose or fructose as substrate. The inhibition of glycolysis was interpreted to be the result of a direct effect on the membrane ATPase system reducing the turnover rate of the cytoplasmic phosphoglycerate kinase reaction.

Ouabain, meralluride, and ethacrynic acid inhibited O_2 uptake by the kidney slices. The inhibition of respiration by ouabain and meralluride could have been mediated through a decrease in ADP formation or by a reduced turnover of glycolytic intermediates.

Ethacrynic acid inhibited respiration at lower levels than that required to inhibit glycolysis. Therefore, mitochondrial respiration could be the primary site of action of ethacrynic acid.

These diuretic agents had no effect on glycolysis or respiration in slices that had been depleted of sodium with choline or lithium. Therefore the effects on energy metabolism in the presence of sodium would appear to be related to an effect on the sodium transport process.

ISOLATED subcellular fractions of kidney tissue have been used to study the relationships between membrane transport processes and intracelluluar metabolism.[1, 2] The cells of the kidney nephrons must actively transport sodium across the limiting cellular membranes on the peritubular side of the cells.[3] These membranes are seen as extensive invaginations into the tubular cells in the proximal and distal segments.[4] A membrane fraction prepared from rat kidney homogenates apparently contained these membranes.[5] The membrane fraction stimulated the phosphoglycerate kinase reaction of a cytoplasmic fraction that contained the necessary enzymes of glycolysis.[1] The kinase reaction apparently was stimulated by the membrane $(Na^+ + K^+)$-ATPase activity.[1, 2] Considerable evidence has accumulated to support the concept that the membrane $(Na^+ + K^+)$-ATPase is an intricate part of the active $Na^+ - K^+$ transport process.[6] This concept has been extended to the membrane ATPase system in renal tubular cells.[2, 5, 7–10]

Whittam and Willis have shown that ouabain, a specific inhibitor of the membrane $(Na^+ + K^+)$-ATPase system, inhibited the accumulation of potassium by kidney slices.[11] Oxygen uptake by the slices was inhibited at the same levels of ouabain

* Present address: Department of Physiology, Cornell University Medical College, New York, N.Y. 10021, U.S.A.

that inhibited potassium accumulation. The data were interpreted to indicate that the reduced ADP formation by the membrane ATPase would diminish the activation of mitochondrial oxidative phosphorylation and oxygen uptake. Whittam and Ager demonstrated that ouabain inhibited glycolysis in erythrocytes, an action which was also attributed to an effect of the glycoside on the membrane ATPase system.[12] Since the glycolytic enzymes are present in high concentrations in the kidney cytoplasm[13] glycolytic intermediates may be involved in the control of cellular energy metabolism related to active sodium transport.

Experiments were designed to determine whether ouabain would also inhibit glycolysis in kidney slices. Kidney cortical slices can metabolize glucose,[13, 14] although the energy derived from glycolysis alone would not be sufficient to maintain intracellular levels of sodium and potassium normally present in kidney cells.[15, 16] Since glycolysis is dependent on the presence of potassium,[17] intracellular levels should be maintained when the effects of transport inhibitors are studied. Intracellular potassium can be maintained by increasing the levels in the incubation medium.[18, 19] Glucose metabolism in kidney slices can be estimated by lactate release into the medium if air is used instead of 100 per cent oxygen.[13] However, under these conditions very little net movement of sodium and potassium occurs,* probably as the result of active movements being countered by a rapid passive leak that is characteristic of kidney slices.[20] Ouabain and meralluride (2×10^{-4} M) inhibited sodium extrusion in rat kidney slices without significantly affecting net potassium movement when slices were incubated in 30 mM potassium Ringer solution in air with glucose as substrate.*

Experiments were also carried out to determine whether meralluride and ethacrynic acid affected the metabolism of kidney slices in a manner similar to that of ouabain. As the level of ouabain or meralluride was increased in the medium, glycolysis and respiration apparently were inhibited simultaneously. However, ethacrynic acid inhibited respiration at a lower concentration than that required to inhibit glycolysis. A preliminary report on these experiments has been published.[21]

EXPERIMENTAL

Slices were prepared from rabbit kidney cortex and whole rat kidney with a Stadie–Riggs microtome after the animals had been sacrificed by a sharp blow to the back of the neck at the base of the skull. The slices were placed in aerated Krebs–Ringer phosphate solution at room temperature for 10–15 min prior to insertion into the Warburg flasks. Krebs–Ringer phosphate (KRP) solution was modified to contain 112 mM NaCl, 4·5 mM KCl, 1·1 mM $MgCl_2$, 2·4 mM $CaCl_2$, 15 mM potassium phosphate buffer (pH 7·4), and 10 mM glucose. The final incubation medium contained 2·8 ml isosmotic KRP, 0·1 ml of 0·3 M glucose, and 0·1 ml H_2O or inhibitor. The center well contained 0·2 ml 10% KOH with fritted filter paper to absorb CO_2. Slices were incubated for 110 min at 37° after a 10-min equilibration period with the manometers open to air. The dry weight of the slices ranged from 10–15 mg per flask. In some experiments slices were preincubated in 0·15 M choline chloride or 0·15 M LiCl for 2·5 hr at 2° to deplete the tissue sodium.

Lactate was measured by the method of Barker and Summerson.[22] Glucose was determined by the glucose oxidase method employed by Krebs *et al.*[23]

* F. Bowman and E. J. Landon, unpublished results.

Meralluride was a gift of Lakeside Laboratories. Ethacrynic acid was generously supplied by Merck, Sharp & Dohme. Ouabain was obtained from Nutritional Biochemical Corp.

RESULTS

Fresh slices of rabbit kidney cortex or slices of whole rat kidney were incubated in modified Krebs–Ringer phosphate (KRP) solution that contained high potassium (30 mM) to maintain intracellular levels.[18, 19] The slide medium was equilibrated with air instead of 100% O_2 to favor glycolysis.[13] These conditions allow the simultaneous measurement of glycolysis and respiration.

When glucose was added as substrate, lactate was formed by rat and rabbit kidney slices and released into the medium (Table 1). These observations are in agreement

TABLE 1. EFFECT OF GLUCOSE ON LACTATE FORMATION AND O_2 UPTAKE BY RAT AND RABBIT KIDNEY SLICES*

	Lactate	O_2
Rat		
No substrate	0·91 ± 0·12	4·62 ± 0·20
Glucose, 10 mM	3·38 ± 0·18†	5·40 ± 0·22
Rabbit		
No substrate	0·61 ± 0·15	2·91 ± 0·23
Glucose, 10 mM	3·67 ± 0·19†	4·22 ± 0·31†

* Mean values of 8 experiments are given as μmole/10 mg dry wt./2 hr ± S.E. Slices (10–15 mg dry wt.) were incubated for 2 hr in 3·0 ml of modified Krebs–Ringer phosphate solution in standard Warburg flasks at 37° in an initial atmosphere of air.

† Significantly different at the 5 per cent level.

with those of Wu[13] when slices were incubated in an atmosphere of air instead of 100% O_2. Glucose did not significantly alter the O_2 uptake in rat kidney slices, but increased the O_2 uptake in rabbit kidney cortical slices (Table 1). Most experiments with diuretic agents were carried out with slices of rabbit kidney cortex.

Ouabain,[5, 7, 9, 10] meralluride,[2, 5, 10] and ethacrynic acid[24] are diuretic agents that have been shown to inhibit the $(Na^+ + K^+)$-ATPase activity or isolated kidney membranes. The effects of various levels of these diuretic agents on glycolysis and respiration of rabbit kidney cortical slices are seen in Fig. 1. Ouabain and meralluride significantly inhibited lactate formation at 10^{-4} M. Ethacrynic acid inhibited lactate formation only at higher levels (5×10^{-4} M). All three diuretics caused a significant decline in O_2 uptake at 10^{-4} M.

Sodium in the slice and the medium was replaced with choline or lithium to determine whether the effects of the diuretics on metabolism would occur in the absence of Na^+ transport. The slices were incubated in 0·15 M choline chloride[25] or in 0·15 M LiCl[26, 27] for 2·5 hr at 2° to reduce tissue Na^+. The NaCl in the modified KRP was also replaced with choline chloride for the incubation at 37° which is also shown in Fig. 1. The replacement of Na^+ with choline resulted in a lower rate of lactate formation and O_2 uptake. These metabolic parameters were not further reduced with

ouabain, meralluride or ethacrynic acid at 1 to 2 $\times$ 10^{-4} M. Essentially the same results were obtained when Na^+ was replaced with Li^+. Lithium is not transported by a ouabain-sensitive process in kidney slices.[27]

Prior incubation of kidney slices in the cold has been repeatedly demonstrated not to reduce the ability of slices to actively transport Na^+ or K^+ when metabolic

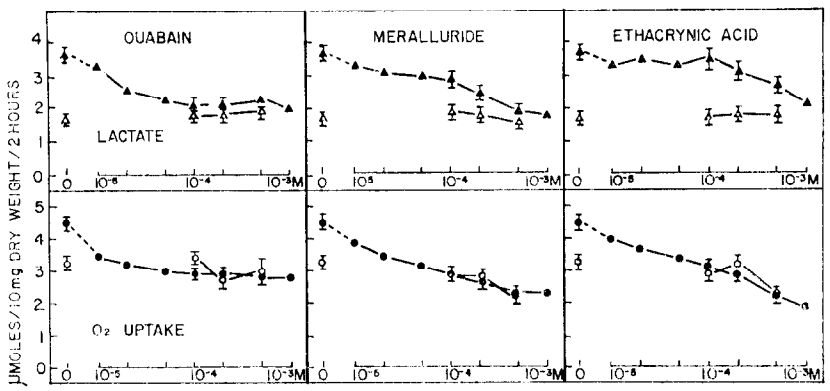

FIG. 1. Effect of ouabain, meralluride, and ethacrynic acid on glycolysis and respiration of slices of rabbit kidney cortex. Experimental conditions were the same as those described in Table 1 for lactate formation (▲ — ▲) and O_2 uptake (● — ●) with glucose as substrate. Tissue sodium was partially depleted by incubating slices in 0·15 M choline chloride for 2·5 hr at 2°. Lactate formation (△ — △) and O_2 uptake (○ — ○) were then determined in slices incubated in modified Krebs–Ringer phosphate solution at 37°, with glucose as substrate and Na^+ replaced with choline. Values for which standard errors are given (vertical bars) represent a mean of 6 experiments. Values without S.E. represent the mean of at least 3 experiments.

substrates were added[15, 16, 18, 19, 26–28] Ouabain inhibited the accumulation of K^+ by slices that had been depleted in the cold.[27, 28] Also, metabolism is apparently unaltered, since the respiration of slices in sodium Ringers solution at 25° was unaffected by prior incubation in LiCl solution in the cold.[27] Tissue K^+ was not depleted when kidney cortical slices were incubated in Na^+-free choline medium at 5° or 38°.[25] Therefore, it was not considered necessary to determine the effect of incubation in the cold *per se* on the $Na^+ - K^+$ transport or metabolic capacity of the slices.

It was not possible to determine the step in glycolysis that was inhibited by ouabain and meralluride from the above data in Fig. 1. However, fructose is known to enter the Embden–Meyerhof pathway at the 3-carbon level, since it is converted to fructose-1-phosphate by fructokinase and then split by the aldolase reaction to dihydroxyacetone phosphate and glyceraldehyde.[29, 30] Therefore, by measuring glucose and lactate formation from fructose, the site of inhibition of glycolysis by transport inhibitors can be localized to the 6-carbon or 3-carbon reactions. The effects of ouabain, meralluride, and ethacrynic acid on fructose metabolism in slices of rabbit kidney cortex are shown in Fig. 2. The diuretics inhibited the conversion of fructose to lactate in a manner similar to that observed with glucose as substrate. The three diuretics had no significant effect on glucose formation; therefore the S.E.M.'s are

not shown. The three diuretic agents also inhbited O_2 uptake when fructose was used as substrate.

The effects of 10^{-4} M ouabain, meralluride, and ethacrynic acid on glycolysis and respiration by rat kidney slices are shown in Table 2. The effects were not qualitatively different from those observed with rabbit kidney cortical slices.

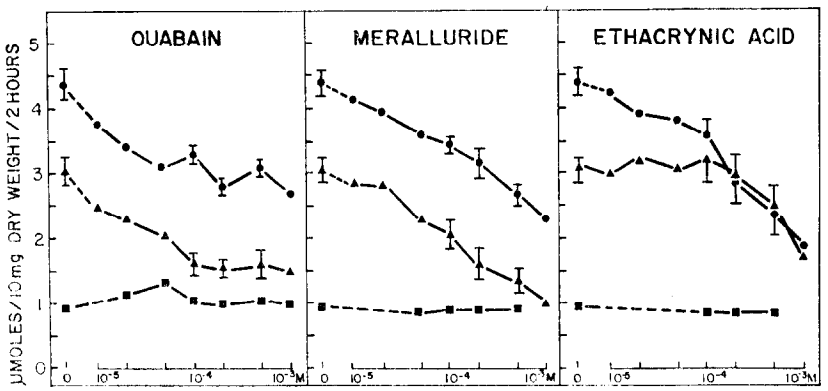

FIG. 2. Effect of ouabain, meralluride, and ethacrynic acid on fructose metabolism by rabbit kidney cortical slices. Experimental conditions were the same as those described in Table 1 except that fructose was substrate and glucose formation (■ — ■) was determined in addition to lactate formation (▲ — ▲) and O_2 uptake (● — ●). The values represent the mean of 6 experiments ± S.E. (vertical bars). Other values represent the mean of at least 3 experiments.

TABLE 2. EFFECT OF OUABAIN, MERALLURIDE, AND ETHACRYNIC ACID ON LACTATE FORMATION AND O_2 UPTAKE BY RAT KIDNEY SLICES*

Inhibitor	Lactate (μmole/10 mg dry wt./2 hr)	O_2
None	3·38 ± 0·18	6·16 ± 0·21
Ouabain	2·60 ± 0·24†	4·86 ± 0·22†
Meralluride	2·66 ± 0·23†	4·96 ± 0·20†
Ethacrynic acid	3·03 ± 0·19	4·41 ± 0·15†

* Experimental conditions were the same as those described in Table 1 with glucose as substrate. Inhibitor level was 10^{-4} M. Values are the mean of 6 experiments ± S.E.

† Significantly different at the 5 per cent level.

DISCUSSION

Recent evidence supports the concept that ouabain[5, 7–10] and mercurial diuretics[2, 5, 10] inhibit renal sodium reabsorption by inhibiting the membrane ($Na^+ + K^+$)-ATPase. The concept of the ATPase system participating in sodium reabsorption is attractive since it readily provides an explanation for the control of renal cellular energy metabolism by sodium transport. If the ADP formed from the ATPase reaction is proportional to the sodium transported and intracelullar energy metabolism depends on ADP levels, then sodium transport could influence energy metabolism by controlling ADP production. The energy requirements for active sodium reabsorption

in the rabbit kidney apparently require complete breakdown of metabolic substrates,[31] which agrees with the observation that oxygen uptake by the kidney is proportional to sodium reabsoption.[32, 33] Therefore, the primary energy-yielding reactions occur in the mitochondria. However, the mechanism of coupling mitochondrial energy metabolism to active transport processes in cell membranes remains to be elucidated.

The existence of high levels of phosphoglycerate kinase in the cytoplasm[13] and the interaction between the membrane ATPase and the kinase[1] suggest that certain glycolytic intermediates may be involved in the transfer of metabolic energy from the mitochondria to the cell membrane (or endoplasmic reticulum).

The present study demonstrates that ouabain inhibits glycolysis and respiration of rabbit and rat kidney slices. Ouabian is not known to inhibit glycolysis of isolated cytoplasm or respiration of isolated mitochondria. Therefore ouabain most likely inhibits these metabolic parameters in slices as a result of an inhibition of the membrane ($Na^+ + K^+$)-ATPase system.

Meralluride inhibited glycolysis and respiration of rabbit and rat kidney slices in a manner similar to that observed with ouabain. Pretreatment of rats with diuretic doses of mercurial diuretics did not inhibit glycolysis of isolated cytoplasm[2] or oxidative phosphorylation of isolated mitochondria,[34] but did reduce the membrane ATPase activity.[2] Therefore meralluride at lower levels apparently inhibits glycolysis and respiration by inhibiting the membrane ($Na^+ + K^+$)-ATPase system.

Ethacrynic acid inhibited respiration at lower levels than that required to inhibit glycolysis in rabbit and rat kidney slices. These data suggest a direct effect on mitochondrial respiration. Higher levels of ethacrynic acid than meralluride were required to inhibit the membrane ($Na^+ + K^+$)-ATPase system.[24] Further work is being carried out to determine the specific site of action of ethacrynic acid at the cellular level.[35]

The concentration of ouabain required to inhibit metabolism in kidney slices is probably higher than those ever reached *in vivo*. However, the kidney slices must accumulate mercurials when the concentration in the medium is 10^{-4} M in order to reach levels comparable to those found in rat and dog kidneys after diuretic doses of chlormerodrin.[36] The level of ethacrynic acid may not reach 10^{-4} M in the kidney *in vivo*.[37]

The use of fructose as substrate provided evidence that ouabain and meralluride inhibited the Embden-Meyerhof pathway at the 3-carbon level. Considering the work with isolated membrane fractions from rat kidney[2] and erythrocytes,[38] the phosphoglycerate kinase reaction would appear to be the step affected by an inhibitor of the membrane ATPase system.

The inhibition of respiration could be the result of a decrease in the supply of ADP to mitochondrial oxidative phosphorylation. Also a decrease in the rate of turnover of glycolytic intermediates may be involved in the inhibition of respiration observed in the present study. The data are consistent with the concept of an intimate relationship between active cation transport, cytoplasmic glycolysis, and mitochondrial respiration in intact kidney cells.

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