The effect of ouabain on metabolic rate in guinea-pigs: estimation of energy cost of sodium pump activity

BY R. SWAMINATHAN, ELLEN L. P. CHAN, LIU YUET SIN, NG SIU KING, NG SUI FUN AND AMY Y. S. CHAN

Department of Chemical Pathology, Prince of Wales Hospital, Shatin, N.T., Hong Kong

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1. The effect of different doses of ouabain, an inhibitor of the sodium pump, or saline (9 g sodium chloride/l; the vehicle) on the metabolic rate of guinea-pigs weighing 500 g was measured by indirect calorimetry for 120 min.

2. Ouabain (0·02–0·07 nmol/g body-weight) decreased the oxygen consumption in a dose-related manner. When higher doses of ouabain (0·10 nmol/g body-weight or greater) were injected the animals were observed to shiver. Ouabain (0·07 nmol/g body-weight) reduced Na^+,K^+-ATPase (*EC* 3 . 6 . 1 . 3) activity of liver, kidney and skeletal muscle by 18·0 (SE 6·6), 21·5 (SE 6·0) and 21·9 (SE 6·8)% respectively. An Eadie-Hofstee plot of percentage decrease in O_2 consumption *v.* percentage inhibition ÷ dose of ouabain showed that maximal inhibition of O_2 consumption was 39·4%.

3. It is concluded that Na pump activity contributed to about 40% of the resting O_2 consumption.

Active transport of sodium and potassium across the cell membrane is an essential process in the maintenance of the intracellular Na and K concentrations. It has been shown that for each ATP molecule hydrolysed three Na ions are pumped out and two K ions are pumped in (Sweadner & Goldin, 1980). The energy cost of this process has been of considerable debate (Milligan & McBride, 1985). Estimates of the cost of Na pump activity have varied from 2 to 45% (Milligan & McBride, 1985). The magnitude of this value has also been reported to vary with the tissue studied. However, even for the same tissue there is very little agreement on the energy cost of the Na pump. For instance, McBride & Milligan (1985*a*) estimated that in sheep the ouabain-sensitive respiration of hepatocytes was about 35%, whereas Clark *et al.* (1982) reported that in rat hepatocytes the ouabain-suppressible oxygen uptake was 2–8%. Similar discrepancies have been reported for muscle tissue (Asano *et al.* 1976; Biron *et al.* 1979; Gregg & Milligan, 1982). Furthermore, there are no estimates for the contribution of the Na pump to the basal or resting metabolic rate in the whole animal. In an in vivo study Saddlier & DeLuise (1986) concluded that the Na pump plays a significant role in the determination of energy turnover. In human volunteers given therapeutic doses of digoxin the resting metabolic rate decreased significantly by 7·6% and leucocyte Na transport by 29%. From these findings it was estimated that the energy cost of the Na pump is at least about 20% of the resting metabolic rate (Swaminathan *et al.* 1982). In order to extend these studies we decided to examine the effect of different doses of ouabain, an effective Na pump inhibitor, on metabolic rate in guinea-pigs. The guinea-pig was selected as the experimental animal because detailed information is available on the distribution of ouabain-binding sites in various tissues of the guinea-pig (Kjeldson *et al.* 1985).

METHODS

Animals

Guinea-pigs weighing 500 g were used in the study. Equal numbers of males and females were used. The animals were housed at 22° and allowed free access to water and laboratory chow up to 1 h before the metabolic rate measurements.

Measurement of oxygen consumption

Metabolic rate was determined by indirect calorimetry in a continuous flow system (Model O_2 ECO, Columbus Instruments, Ohio, USA). The system consists of a measuring chamber ($200 \times 180 \times 400$ mm) and a control unit containing circulating pumps, a flowmeter and an O_2 sensor. Ambient air was pumped (pump 1) into the chambers via the input flowmeter at a rate of 2 litres/min. Air from the measuring chamber was removed by another pump (pump 2) via an air valve. The air valve can be switched manually or automatically to draw air from the measuring chamber or draw atmospheric air. The flow rate into the chamber was always kept higher than that leaving the chamber so as to prevent any extraneous air leaking into the chamber. The air coming from pump 2 was dried and carbon dioxide removed before a sample was passed into the O_2 sensor (electrochemical analyser) (Columbus Instruments, Ohio, USA) for measurement of O_2 concentration via a flowmeter (at the rate of 1 litre/min). We used a timer device to switch the valve so that the O_2 sensor measured the O_2 concentration in the atmospheric air for 5 min followed by O_2 concentration in the air coming from the chamber for 5 min. This cycle was repeated automatically.

The flow rates were found to be stable for at least 6 h. The output from the sensor was recorded on a chart and this showed that mixing was complete as shown by steady values for 5 min.

The temperature of the measuring chamber was the same as room temperature and all measurements were made at the ambient temperature of the room which was always maintained at 22° by the central air conditioning of the hospital.

The sensor was calibrated using a gas mixture of known composition (20·5% oxygen and 79·5% nitrogen). The precision of the instrument, assessed by using a small burner (Pocketwarmer, Model BG-307E, National, Matsushita Electrical Trading Co., Japan), was found to be 3%.

The O_2 consumption of the animal was calculated from the formula:

$$O_2 \text{ consumption} = \text{Inflow rate} \times \frac{(X_{in}-X_{out})}{(1-X_{out})},$$

where X_{in} and X_{out} are the proportions of O_2 in the air entering and leaving the cage respectively.

Experimental procedures

All experiments were done with pairs of animals, the animals being paired according to weight and sex. One animal from each pair received an intraperitoneal injection of saline (9 g sodium chloride/l) which was used as the vehicle and the other received an intraperitoneal injection of ouabain (0·02–0·30 nmol/g body-weight). The animals were then placed in the measuring chamber. Different sets of six animals were used for each dose of ouabain.

The O_2 consumption rates of males (mean 1·42 (SE 0·069) litres/kg per h) and females (mean 1·40 (SE 0·101) litres/kg per h) were similar and the response to ouabain was also similar in the two sexes. At 80 min after intraperitoneal injection of ouabain (0·07 nmol/g body-weight) the O_2 consumption rate in males was 1·14 (SE 0·122) litres/kg per h and in the females was 1·09 (SE 0·119) litres/kg per h. Therefore, in the results reported, values for male and female guinea-pigs were pooled.

In a second experiment, two groups of guinea-pigs (not previously exposed to ouabain), seven in each group, were used to determine the effect of ouabain on Na^+,K^+-ATPase (*EC* 3 . 6 . 1 . 3). One group was injected intraperitoneally with 0·07 nmol ouabain/g body-

weight and the other with saline. The animals were bled by cardiac puncture 60 min after injection, and were then killed and tissue samples (liver, kidney, muscle and heart) were taken, frozen immediately in liquid nitrogen and stored at −70° until assay. Blood samples were centrifuged, the plasma was separated and the erythrocytes used for the measurement of Na^+,K^+-ATPase activity.

To measure Na^+,K^+-ATPase activity, tissues were thawed just before use and about 0·5 g (wet weight) of each of the tissue samples was minced and homogenized in 5 ml 0·25 M-sucrose, 1·25 mM-EGTA and 30 mM-Tris, pH 7·4, using a Teflon–glass homogenizer in an ice-bath. The homogenate was filtered through one layer of gauze. The filtered suspension (0·1 ml) was incubated with 0·8 ml incubation medium (NaCl 100 mM, KCl 10 mM, $MgCl_2$ 5 mM, Tris 50 mM, sodium azide 5 mM, EDTA 1·0 mM, pH 7·4) for 5 min in a water-bath at 37°. The reaction was started by the addition of 0·1 ml 50 mM-ATP (final concentration 5 mM-ATP). After 30 min the reaction was stopped by the addition of 1 ml cold trichloroacetic acid solution (300 g/l). The mixture was centrifuged and the phosphate concentration of the supernatant fraction was measured by the method of Daly & Ertingshausen (1972). The protein concentration of the homogenate was determined by the method of Lowry *et al.* (1951). The phosphate and protein concentrations were measured in a centrifugal analyser (Cobas-Bio®; Roche Diagnostics, Basle, Switzerland). The Na^+,K^+-ATPase activity was determined as the difference in activity in the absence or presence of ouabain (10^{-3} mol/l).

To determine erythrocyte Na^+,K^+-ATPase activity, packed erythrocytes were haemolysed by freezing and thawing three times and the ATPase activity determined as described previously (Arumanayagam *et al.* 1987). A portion (0·1 ml) of the haemolysate was incubated in a buffer solution, at a final concentration (mmol/l): ATP 2, NaCl 100, KCl 25, $MgCl_2$ 3, EGTA 1. The haemolysate was incubated in duplicate, ouabain (10^{-2} mol/l) being added to one sample to inhibit Na^+,K^+-ATPase activity. The mixtures were incubated at 37° for 30 min, and the reaction was stopped by the addition of ice-cold trichloroacetic acid (10^{-1} mol/l). The reaction tubes were centrifuged and the phosphate concentration in the supernatant fraction was determined as described previously. The haemoglobin (Hb) concentration in the haemolysate was determined by the cyanmethaemoglobin method using a centrifugal analyser. Na^+,K^+-ATPase activity was expressed as pmol phosphate released per Hb. The effect of ouabain was expressed as percentage change compared with the mean activity of the control group.

RESULTS

Fig. 1 shows the effect of 0·07 nmol ouabain/g body-weight or an equal volume of saline on the O_2 consumption. When compared with the animals injected with saline, the O_2 consumption rate was found to decrease significantly at 80 min and it remained low until the end of the experiment (120 min) in ouabain-treated animals.

Fig. 2 shows the effect of different doses of ouabain on the O_2 consumption rate, 80–120 min after injection, expressed as a percentage of the control value. With increasing dose of ouabain there was a linear decrease in O_2 consumption. In an initial experiment it was observed that when the dose of ouabain was 0·1 nmol/g body-weight or greater, O_2 consumption either did not change or increased. At these higher doses the animals started to shiver, and at 0·30 nmol/g body-weight the animals died. These effects were taken to represent secondary effects (see Discussion). Table 1 shows the effect of 0·07 nmol ouabain/g body-weight on Na^+,K^+-ATPase of different tissues. There were significant reductions in the Na^+,K^+-ATPase activity of liver, kidney and muscle, the effect being most pronounced in kidney and muscle.

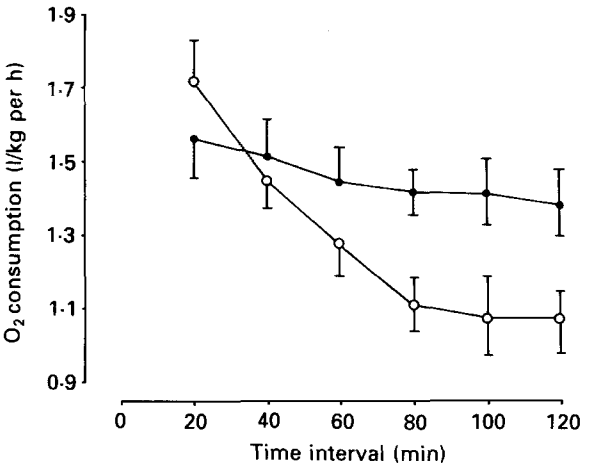

Fig. 1. The effect of 0·07 nmol ouabain/g body-weight (○) and saline (9 g sodium chloride/l) (●) on oxygen consumption rate in guinea-pigs. Points are means, with their standard errors represented by vertical bars, for six animals. For details of procedures, see p. 468.

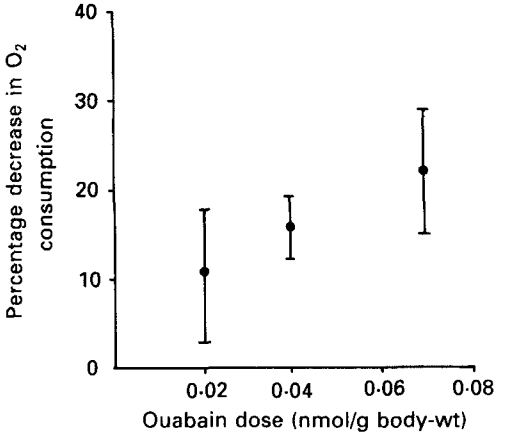

Fig. 2. Percentage change in oxygen consumption 80–120 min after intraperitoneal injection of different doses of ouabain. At each dose six animals were used. Points are means, with their standard errors represented by vertical bars. For details of procedures, see p. 468.

When the results in Fig. 2 were analysed by an Eadie-Hofstee plot (Fig. 3), where percentage decrease in O_2 consumption *v.* percentage inhibition ÷ ouabain dose is plotted (Gstraunthaler *et al.* 1985), the maximal inhibition of O_2 consumption was calculated to be 39·4%. The dose of ouabain required to cause 50% of the maximal inhibition of O_2 consumption (K_i) was 0·057 nmol/g body-weight.

DISCUSSION

In the present study, in order to determine the energy cost of Na transport to the whole animal, we have used an in vivo experimental model. The results show that injection of ouabain caused 10–22% inhibition of resting O_2 consumption. This suggests that Na transport contributes significantly to total energy cost in guinea-pigs. Lin *et al.* (1981)

Table 1. *The effect of intraperitoneal injection of 0·07 nmol ouabain/g body-weight on Na^+,K^+-ATPase (EC 3.6.1.3) activity (μmol inorganic phosphate released/mg protein per h) in different tissues in guinea-pigs*

(Mean values with their standard errors for seven animals)

	Na^+,K^+-ATPase activity					
	Saline (9 g sodium chloride/l)-treated		Ouabain-treated		Percentage change in Na^+,K^+-ATPase activity†	
	Mean	SE	Mean	SE	Mean	SE
Erythrocytes‡	12·0	0·71	10·8	0·80	−10·0	6·7
Liver	0·293	0·022	0·240	0·022	−18·0*	6·6
Kidney	2·28	0·126	1·79	0·136	−21·5***	6·0
Heart	0·406	0·026	0·365	0·025	−10·1	6·2
Muscle	0·282	0·039	0·220	0·019	−21·9**	6·8

Statistical significance of difference from zero by *t* test: * $P < 0·05$, ** $P < 0·025$, *** $P < 0·01$.

† Changes in Na^+,K^+-ATPase activity are expressed as the percentage change compared with the mean values for the saline-treated control group.

‡ μmol phosphate/g haemoglobin per h.

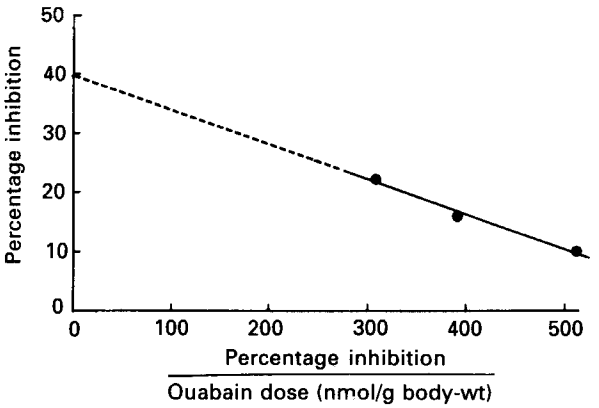

Fig. 3. Eadie–Hofstee plot of ouabain-induced inhibition of oxygen consumption rate in guinea-pigs. For details of procedures, see p. 468.

reported that injection of ouabain into mice decreased O_2 consumption by 20–25%, which is in agreement with our findings.

Previous in vitro studies on the energy cost of the Na pump have been made on isolated tissues or cells and the results have been conflicting (Asano *et al.* 1976; Biron *et al.* 1979; Guernsey & Morishige, 1979; Gstraunthaler *et al.* 1985; Milligan & McBride, 1985). Some of these conflicting results could be due to differences in the concentration of ouabain used. For example, in some of the in vitro studies, where a relatively high concentration of ouabain was used to determine the contribution of the Na pump, the inhibition of O_2 consumption by ouabain was relatively small (Biron *et al.* 1979; Clark *et al.* 1982; Wardlaw, 1986). In studies where a lower concentration of ouabain had been used, 20–40% inhibition of O_2 consumption was observed (Gregg & Milligan, 1982; McBride & Milligan,

1985*a*, *b*). We therefore suggest that a wide range of ouabain concentrations should be used to evaluate the ouabain-suppressible O_2 consumption.

The use of ouabain to assess the contribution of Na pump activity to energy cost has been criticized (Himms-Hagen, 1976). It has been suggested that ouabain may inhibit enzymes other than Na^+,K^+-ATPase, and that the fall in O_2 consumption may be secondary to impairment of mitochondrial function, or due to an alteration in the intracellular Na/K concentration induced by inhibition of the Na pump. However, no enzyme system, including mitochondrial ATPases, has been shown to be inhibited by digitalis glycosides (Smith & Edelman, 1979). It is also unlikely that the observed effects are secondary to changes in the intracellular concentration of Na/K. In preliminary experiments, we failed to find any difference in the Na or K contents of liver, muscle, kidney and heart, 60 min after injection of 0·07 nmol ouabain/kg (R. Swaminathan and E. Chan, unpublished results). Lin *et al.* (1981), similarly, did not find any significant effect of ouabain on the Na/K concentration in liver, kidney or skeletal muscle. At high concentrations ouabain may cause changes in intracellular ions, including calcium (Wollenberger, 1947), and this increase in intracellular Ca may have secondary effects, such as stimulation of muscle contraction, and these may lead to a negligible decrease or even an increase in O_2 consumption. The relatively low inhibition of O_2 consumption reported by some investigators may be explained by this phenomenon (see p. 471). We did observe 'shivering' when 0·1 nmol ouabain/g body-weight or more was injected. Although the effect of ouabain on O_2 consumption is likely to be specific, we cannot exclude other effects of ouabain on autonomic function (e.g. cardiovascular effects) or behaviour. Therefore, we can only draw tentative conclusions about the contribution of the Na pump to basal metabolic rate.

This in vivo study shows that the maximum ouabain-inhibitable O_2 consumption is 40% in the guinea-pig, which is of the same order of magnitude as reported by others (Gregg & Milligan, 1982; McBride & Milligan, 1985*a*, *b*; Milligan & McBride, 1985), and is in agreement with our previous in vivo study in man (Swaminathan *et al.* 1982). However, in in vivo studies, the extent of binding of ouabain by different tissues may be variable. It is likely that a great proportion of the ouabain will bind to muscle (Kjeldson *et al.* 1985). Kjeldson *et al.* (1985) injected various doses of ouabain into guinea-pigs and estimated the percentage of binding sites occupied by ouabain in muscle and heart. It can be estimated from their findings that at a dose of 0·07 nmol/g body-weight, less than 20% of binding sites in heart and muscle are occupied. This dose of ouabain caused a 22% decrease in O_2 consumption. Therefore, if all the binding sites were to be occupied the decrease in O_2 consumption would be greater than 100%. Thus, it is possible that ouabain decreased O_2 consumption by mechanisms other than inhibition of the Na pump. An alternative explanation is that ouabain-binding studies (at high concentrations of ouabain) may measure all available Na pump units and some of them may be 'inactive'. Further studies are in progress to address these possibilities.

We conclude that ouabain causes a dose-related inhibition of O_2 consumption in guinea-pigs and the findings suggest that Na pump activity contributes approximately 40% of resting energy expenditure.

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