No major thermogenic role for (Na⁺ + K⁺)-dependent adenosine triphosphatase apparent in hepatocytes from hyperthyroid rats

Dallas G. CLARK,* Menno BRINKMAN,* Owen H. FILSELL,* Simon J. LEWIS† and Michael N. BERRY†

*CSIRO Division of Human Nutrition, Kintore Avenue, Adelaide, South Australia 5000, Australia, and

†Clinical Biochemistry Unit, Flinders University School of Medicine, Bedford Park, South Australia 5042, Australia

(Received 14 September 1981/Accepted 6 November 1981)

(Na⁺ + K⁺)-dependent ATPase activity, heat production and oxygen consumption were increased by 59%, 62% and 75% respectively in hepatocytes from tri-iodothyronine-treated rats. Ouabain at concentrations of 1 and 10 mM decreased oxygen uptake by 2–8% in hepatocytes from euthyroid rats and by 5–15% in hepatocytes from hyperthyroid animals. Heat output was decreased by 4–9% with the glycoside in isolated liver parenchymal cells from the control animals and by 11% in the cells from the tri-iodothyronine-treated animals. These results do not support the hypothesis that hepatic (Na⁺ + K⁺)-ATPase plays a major role in increased heat production in hepatocytes from hyperthyroid rats.

The role of the (Na⁺ + K⁺)-ATPase in cellular thermogenesis has received considerable attention during the past 15 years. One of the main reasons for this is the uncertainty concerning the degree of energy expenditure associated with the maintenance of intracellular Na⁺ and K⁺ concentrations by the Na⁺ pump. This has been suggested to range from 5–6% (Crone, 1966; Chinet *et al.*, 1977; Folke & Sestoft, 1977) to 30–60% of the basal cellular energy requirements (Clausen, 1966; Ismail-Beigi & Edelman, 1970; Edelman, 1976; Guernsey & Morishige, 1979). Assessments of the contribution of this enzyme system to thermogenesis in isolated tissues from hyperthyroid animals has produced equally diverse results: these vary from 5–6% (Folke & Sestoft, 1977) to 45–90% (Ismail-Beigi & Edelman, 1970; Asano *et al.*, 1976).

In the present study we have attempted to determine more precisely the role of (Na⁺ + K⁺)-ATPase in hepatic thermogenesis and thyroid-hormone-induced thermogenesis: to this end we have measured heat production, oxygen consumption and (Na⁺ + K⁺)-ATPase activities in isolated liver parenchymal cells from normal and tri-iodothyronine-treated rats in the presence and absence of 1 mM and 10 mM-ouabain.

Abbreviation used: (Na⁺ + K⁺)-ATPase, (Na⁺ + K⁺)-dependent adenosine triphosphatase (EC 3.6.1.3).

Materials and methods

Collagenase (type II) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Albumin (crystalline bovine, fatty-acid-poor), 3,3',5-tri-iodo-L-thyronine (sodium salt) and vanadium-free ATP (disodium salt) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Ouabain was from Boehringer/Mannheim Pty., Mount Waverley, Victoria 3149, Australia, and Triton X-100 was manufactured by Rohm and Haas Co., Philadelphia, PA, U.S.A.

Animals

Adult male Hooded Wistar rats (240–260 g body wt.) were injected subcutaneously with either 3,3',5-tri-iodo-L-thyronine (50 µg/100 g body wt.) or the diluting medium (0.5 mM-NaOH; Ismail-Beigi & Edelman, 1971) on three alternate days and were used 24 h after the final injection.

Preparation of hepatocytes

Isolated liver parenchymal cells were prepared from rats fed *ad libitum* by the method of Berry & Friend (1969), except that the perfusion buffer was Ca²⁺-free saline serum substitute (Krebs, 1950), hyaluronidase was omitted and the final concentration of collagenase was 0.04% (w/v). The hepatocytes were washed and suspended in Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit,

1932) containing 10 mM-glucose and 2.5% (w/v) fatty-acid-poor bovine serum albumin.

Heat production and O_2 consumption

The heat produced by isolated hepatocytes was measured in a modified LKB model 10700 batch-type microcalorimeter as described previously (Jarrett *et al.*, 1979; Clark *et al.*, 1979), and O_2 consumption was determined polarographically in a Yellow Springs Instrument model 53 oxygen electrode assembly (Jarrett *et al.*, 1979).

Measurement of cellular integrity

Lactate dehydrogenase (EC 1.1.1.27) leakage and Trypan Blue uptake were measured before and after each incubation in the microcalorimeter.

Preparation of subcellular fractions

All procedures were performed at 0–4°C. Hepatocytes (approx. 100 mg wet wt.) were suspended in 1.0 ml of homogenizing medium (0.25 M-sucrose, 1.25 mM-EGTA and 10 mM-Tris, pH 7.0; Ismail-Beigi & Edelman, 1971) in the presence of 0.5% (w/v) Triton X-100 and homogenized with 20 strokes of a Blaessig glass homogenizer. The homogenate was centrifuged at 50 g for 1.5 min and the supernatant diluted 1:4 with incubation medium (100 mM-glycylglycine, 2 mM-MgSO₄, 0.2 mM-EDTA, 80 mM-NaCl and 20 mM-KCl, adjusted to pH 7.6 with Tris; Filsell & Jarrett, 1965).

Determination of $(Na^+ + K^+)$ -ATPase

Incubations were performed with 0.10 ml of enzyme essentially as described by Filsell & Jarrett (1965), except that 5 mM-NaN₃ was included in the incubation (Ismail-Beigi & Edelman, 1971) and vanadium-free ATP was used as substrate (Cantley *et al.*, 1978). P_i was determined in the HClO₄ supernatants (Allen, 1940), and the method of Lowry *et al.*, (1951) was used to assay protein in the diluted enzyme mixture. Total ATPase activity in liver homogenates is assumed to represent the sum of two separate activities, $(Na^+ + K^+)$ -ATPase and Mg²⁺-dependent ATPase (Ismail-Beigi & Edelman, 1971). The contribution of the two enzymes to ATP hydrolysis can be distinguished with ouabain: this inhibits $(Na^+ + K^+)$ -ATPase, but not Mg²⁺-ATPase (Asano *et al.*, 1976).

Determination of intracellular Na^+ and K^+ concentrations

Intracellular concentrations of Na^+ and K^+ were determined by the [¹⁴C]inulin method of Krebs *et al.* (1974), except that the concentrations of these ions were measured with a Beckman Astra-8 Automated Stat-routine analyser.

Statistical methods

The statistical significance of differences between experimental groups was determined by the analysis of variance (Brownlee, 1949). In some experiments Student's *t* test was applied to assess significant differences. A *P* value of 0.05 or less was taken as the criterion for statistical significance.

Results and discussion

When ouabain is employed as an inhibitor of $(Na^+ + K^+)$ -ATPase, whether in whole tissues, tissue slices, isolated cells or in cell-free extracts, the glycoside is usually present at a concentration of 1 mM (Ismail-Beigi & Edelman, 1970; Chinet *et al.*, 1977; Horwitz & Eaton, 1977). Preliminary investigations with hepatocytes prepared from tri-iodothyronine-treated rats (results not shown) revealed that ouabain, at this concentration, decreased oxygen consumption and heat production by only 10–12% (cf. Ismail-Beigi & Edelman, 1971). In an attempt to ensure that $(Na^+ + K^+)$ -ATPase was completely inhibited, subsequent experiments were performed with ouabain at concentrations of both 1 and 10 mM (Folke & Sestoft, 1977).

Effect of thyroid status on $(Na^+ + K^+)$ -ATPase

The activities of $(Na^+ + K^+)$ -, Mg²⁺- and total ATPase in cell-free extracts of isolated liver parenchymal cells from euthyroid and hyperthyroid rats (see the Materials and methods section) are shown in Table 1. Subcutaneous injections of tri-iodothyronine on 3 alternate days elicited a 30% increase in the total-ATPase activity (*P* < 0.01). Most of this increase was due to a 60–65% increment in the activity of $(Na^+ + K^+)$ -ATPase (*P* < 0.05 with 1 mM-ouabain; *P* < 0.001 with 10 mM-ouabain). The activity of Mg²⁺-ATPase was increased by 20% (*P* < 0.01) in the presence of 1 mM-ouabain, but by less than 10% (not significant) with 10 mM-ouabain (Table 1); this result indicates that ouabain, at a concentration of 1 mM, might not completely inhibit $(Na^+ + K^+)$ -ATPase in our assay system. The activity of the Mg²⁺-dependent enzyme in the cell-free hepatocyte extracts (Table 1) was 18–35% less than that reported by Ismail-Beigi & Edelman (1971) for whole liver homogenates, whereas the $(Na^+ + K^+)$ -ATPase was between 2.5- and 4.5-fold higher in the homogenized isolated liver cell preparation. In spite of these differences, the percentage increases in $(Na^+ + K^+)$ -ATPase and Mg²⁺-ATPase after tri-iodothyronine treatment were very similar to those obtained with the intact liver (Ismail-Beigi & Edelman, 1971).

Effect of ouabain and thyroid status on O_2 consumption and heat production

More substantial evidence for the hypothesis

Table 1. *Effect of thyroid status on total ATPase, (Na⁺ + K⁺)-ATPase and Mg²⁺-ATPase activities in hepatocytes from euthyroid and tri-iodothyronine-treated rats*

Total and ouabain-suppressible ATPase activities were measured in membrane-rich fractions of hepatocytes prepared from either the euthyroid or tri-iodothyronine-treated rats (initially 240–260 g body wt.) as described in the Materials and methods section. (Na⁺ + K⁺)-ATPase (*A* – *B*) activity (ouabain-suppressible) was calculated as the difference between the activity of the complete system [(Na⁺ + K⁺ + Mg²⁺)-ATPase; *A*] minus that observed in the presence of either 1 mM- or 10 mM-ouabain (Mg²⁺-ATPase; *B*). Values (in $\mu\text{mol of P}_i/\text{h per mg of protein}$) are shown as means $\pm$ s.e.m. for five cell preparations. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 for enzyme activities of hepatic membranes from euthyroid compared with appropriate tri-iodothyronine-treated rats; ns, not significant (*P* > 0.05).

Tri-iodothyronine treatment	Ouabain (mM)	Total ATPase (<i>A</i>)	Mg ²⁺ -ATPase (<i>B</i>)	(Na ⁺ + K ⁺)-ATPase (<i>A</i> – <i>B</i>)
–	1	7.10 $\pm$ 0.49 **	5.10 $\pm$ 0.17 **	2.00 $\pm$ 0.46 *
+	1	9.27 $\pm$ 0.16	6.10 $\pm$ 0.22	3.17 $\pm$ 0.31
–	10	7.10 $\pm$ 0.49 **	4.38 $\pm$ 0.41 ns	2.72 $\pm$ 0.18 ***
+	10	9.27 $\pm$ 0.16	4.77 $\pm$ 0.26	4.50 $\pm$ 0.31

that the Na⁺ pump plays a major role in cellular thermogenesis and in thyroid-hormone-induced thermogenesis has been obtained from comparative measurements of total O₂ consumption and ouabain-suppressible O₂ consumption in liver, muscle and kidney tissue from euthyroid and hyperthyroid animals (Ismail-Beigi & Edelman, 1971; Guernsey & Morishige, 1979). The former workers have shown that during the transition from the euthyroid to the hyperthyroid state total O₂ uptake was increased by 10–25% in kidney slices, 25–35% in quarter diaphragm segments (Asano *et al.*, 1976) and by over 50% in rat liver slices (Ismail-Beigi & Edelman, 1971). Although other research groups have reported similar increases in total O₂ consumption after injections of tri-iodothyronine or thyroxine, some of these investigators have been unable to demonstrate any marked inhibition of oxidative metabolism in control or hyperthyroid animals when the (Na⁺ + K⁺)-ATPase system was inhibited (Crone, 1966; Chinot *et al.*, 1977; Folke & Sestoft, 1977). If the maintenance of intracellular Na⁺ and K⁺ concentrations utilizes substantial amounts of ATP, then measurements of cellular heat production in both euthyroid and hyperthyroid animals, in the presence and absence of Na⁺, or specific Na⁺-pump inhibitors, should indicate the contribution of this enzyme system to total energy utilization by the cell.

The effects of 1 mM- and 10 mM-ouabain on O₂ uptake and heat production in isolated hepatocytes from control and tri-iodothyronine-treated rats are shown in Table 2. The hormonal treatment increased O₂ consumption by 8.5 $\mu\text{mol of O}_2/\text{min per g dry wt.}$ of cells and heat production by 4 J/min per g dry wt.: both these increases, 75% for the consumption of O₂ and 62% for the production of heat, were highly significant statistically (*P* < 0.001). The

specific (Na⁺ + K⁺)-ATPase inhibitor, ouabain, at a concentration of 1 mM, decreased O₂ uptake by 8% in hepatocytes from euthyroid rats and by 15% in those from the hyperthyroid animals. Only the latter change was statistically significant (*P* < 0.01). A 10-fold increase in the concentration of the cardiac glycoside had even less effect on the rate of O₂ consumption. In this case (Table 2) O₂ uptake was apparently decreased by only 2% (not significant) in the control hepatocytes and by 6% (not significant) in the cells from the tri-iodothyronine-treated rats.

Simultaneous microcalorimetric measurements of heat production in hepatocytes from the euthyroid animals demonstrated that this was decreased by approx. 5% (not significant) in the presence of 1 mM-ouabain and by 9% (*P* < 0.01) with 10 mM-ouabain (Table 2). In liver cells from tri-iodothyronine-treated rats 1 mM-ouabain inhibited heat output by 9% (*P* < 0.01) and 10 mM-ouabain decreased heat production by 11% (*P* < 0.001). These results show that the (Na⁺ + K⁺)-ATPase is responsible for only a small proportion of the increase in O₂ consumption and heat production which resulted from tri-iodothyronine treatment (Table 2). These results contrast with those of Ismail-Beigi & Edelman (1971), who claimed that up to 90% of the increase in O₂ uptake in rat liver slices that results from tri-iodothyronine treatment was directly attributable to increased Na⁺-pump activity.

The intracellular concentrations of Na⁺ and K⁺ in hepatocytes from control rats were 23.3 $\pm$ 6.04 (5) and 96.0 $\pm$ 3.34 (5) $\mu\text{mol/g wet wt.}$ respectively. These values were reversed after incubation with 1 mM-ouabain, to 91.3 $\pm$ 2.5 (5) and 23.0 $\pm$ 5.3 (5) $\mu\text{mol/g wet wt.}$ Very similar results were obtained with hepatocytes from hyperthyroid rats. In this case the internal concentration of Na⁺ was increased from 18.0 $\pm$ 2.0 (5) to 84.7 $\pm$ 2.3 (5) by

Table 2. *Effect of ouabain on O₂ uptake and heat production by hepatocytes from euthyroid and tri-iodothyronine-treated rats fed ad libitum*

Isolated hepatocytes were prepared from fed euthyroid (268 ± 9.3 g body wt.) and tri-iodothyronine-treated rats (236 ± 8.5 g body wt.) as described in the Materials and methods section. The cells were diluted with Krebs–Henseleit bicarbonate-buffered saline (Krebs & Henseleit, 1932) containing 10 mM-glucose and 2.5% (w/v) fatty-acid poor bovine serum albumin to give approx. 10^6 cells/ml. After pre-equilibration of the cell suspension, with the appropriate additions, at 37°C for 3 min, 12 ml was transferred to the microcalorimeter reaction vessel and 6.0 ml to the oxygen-electrode assembly. Recordings of thermopile output (μ V) and O₂ concentration were taken for 15 min after an additional 10 min equilibration period (Jarrett *et al.*, 1979). Values are shown as means $\pm$ S.E.M., for four cell preparations. ** $P < 0.01$, *** $P < 0.001$ for hepatocytes from control compared with ouabain-inhibited cells (ns, not significant; $P > 0.05$). P values for hepatocytes from euthyroid cells compared with corresponding hyperthyroid cells are shown in parentheses.

Additions to medium	Euthyroid			Hyperthyroid		
	O ₂ uptake (μ mol/min per g dry wt.)	Heat output (J/min per g dry wt.)	Heat output O ₂ uptake (J/ μ mol)	O ₂ uptake (μ mol/min per g dry wt.)	Heat output (J/min per g dry wt.)	Heat output O ₂ uptake (J/ μ mol)
None	11.3 ± 0.75 ns	6.42 ± 0.05 ns	0.57 ± 0.04 ns	19.8 ± 1.1 (***) **	10.4 ± 0.13 (***) **	0.53 ± 0.04 (ns) ns
1 mM-ouabain	10.4 ± 0.69 ns	6.14 ± 0.13 **	0.59 ± 0.03 ns	16.8 ± 0.73 (***) ns	9.47 ± 0.13 (***) ***	0.56 ± 0.03 (ns) ns
10 mM-ouabain	11.05 ± 1.14	5.85 ± 0.11	0.53 ± 0.04	18.7 ± 1.45 (**)	9.28 ± 0.10 (***)	0.50 ± 0.04 (ns)

incubation with 1 mM-ouabain, and intracellular K⁺ was decreased from 91.4 ± 5.6 (5) to 29.7 ± 4.6 (5) μ mol/g wet wt. These results, when coupled with those of Table 2, demonstrate that 1 mM-ouabain does inhibit (Na⁺ + K⁺)-ATPase in isolated rat liver parenchymal cells and that this concentration of the inhibitor is suitable for studies with hepatocytes.

On balance it is apparent that the (Na⁺ + K⁺)-ATPase plays a minor role in the increased energy utilization of hepatocytes from tri-iodothyronine-treated rats. Whether this is a direct response, as suggested by some workers, or is a consequence of other metabolic changes is not apparent from the present results. In hepatocytes from euthyroid animals, leakage of lactate dehydrogenase (EC 1.1.1.27) was $9.5 \pm 1.4\%$, whereas that in the cells from hyperthyroid rats was $16.7 \pm 3.2\%$ ($P < 0.01$). This suggests that some of the ouabain-suppressible O₂ uptake and heat production can be attributed to increased operation of (Na⁺ + K⁺)-ATPase in damaged cells rather than from thyroid-hormone-induced thermogenesis. This finding may help to explain some of the effects observed by Ismail-Beigi & Edelman (1971) in rat liver slices, but it does not indicate why ouabain completely inhibited the large increase in O₂ consumption which resulted after the tri-iodothyronine treatment of their rats.

It is well known that liver slices undergo swelling when incubated in iso-osmotic media (Leaf, 1956). Furthermore many metabolic processes which are characteristic of the intact liver cannot be reproduced in these preparations (Hems *et al.*, 1966). It

seems probable that tissues from hyperthyroid animals, because of their increased metabolic rate, are likely to be very sensitive to hypoxia and other insults which result from the preparation and incubation of tissue slices. Thus data on the ouabain-sensitivity of these preparations may not be readily extrapolated to more intact preparations such as the perfused liver (Folke & Sestoft, 1977) and the isolated hepatocytes used in this investigation.

While this work was in preparation, a similar study on the effects of thyroid hormones and the energetics of active Na⁺ and K⁺ transport in mammalian skeletal muscle was published (Biron *et al.*, 1979). These researchers found that the steady-state heat-production rate was increased by about 30% during the transition from the hypothyroid to the euthyroid state and by about 45% in the transition to the hyperthyroid state. In addition they showed that 1 mM-ouabain decreased heat production by 6–8% in soleus muscle from all three groups of rats.

We are indebted to Dr. R. M. Smith for his helpful comments and criticisms of this work. Intracellular Na⁺ and K⁺ concentrations were determined in the Routine Diagnostic Laboratory, Clinical Biochemistry Unit, Flinders University School of Medicine.

References

Allen, R. J. L. (1940) *Biochem. J.* **34**, 858–865

- Asano, Y., Liberman, U. A. & Edelman, I. S. (1976) *J. Clin. Invest.* **57**, 368–379
- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- Biron, R., Burger, A., Chinnet, A., Clausen, T. & Dubois-Ferrière, R. (1979) *J. Physiol. (London)* **297**, 47–60
- Brownlee, K. A. (1949) *Industrial Experimentation*, 4th edn., p. 74, H.M. Stationery Office, London
- Cantley, L. C., Cantley, L. G. & Josephson, L. (1978) *J. Biol. Chem.* **253**, 7361–7368
- Chinnet, A., Clausen, T. & Girardier, L. (1977) *J. Physiol. (London)* **265**, 43–61
- Clark, D. G., Filsell, O. H. & Topping, D. L. (1979) *Biochem. J.* **184**, 501–507
- Clausen, T. (1966) *Biochim. Biophys. Acta* **120**, 361–368
- Crone, C. (1966) *Acta Physiol. Scand.* **68**, 105–107
- Edelman, I. S. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 2180–2184
- Filsell, O. H. & Jarrett, I. G. (1965) *Biochem. J.* **97**, 479–484
- Folke, M. & Sestoft, L. (1977) *J. Physiol. (London)* **269**, 407–419
- Guernsey, D. L. & Morishige, W. K. (1979) *Metab. Clin. Exp.* **28**, 629–632
- Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966) *Biochem. J.* **101**, 284–292
- Horwitz, B. A. & Eaton, M. (1977) *J. Appl. Physiol.* **42**, 150–153
- Ismail-Beigi, F. & Edelman, I. S. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1071–1078
- Ismail-Beigi, F. & Edelman, I. S. (1971) *J. Gen. Physiol.* **57**, 710–722
- Jarrett, I. G., Clark, D. G., Filsell, O. H., Harvey, J. W. & Clark, M. G. (1979) *Biochem. J.* **180**, 631–638
- Krebs, H. A. (1950) *Biochim. Biophys. Acta* **67**, 104–137
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Krebs, H. A., Cornell, N. W., Lund, P. & Hems, R. (1974) *Alfred Benzon Symp.* **6**, 726–750
- Leaf, A. (1956) *Biochem. J.* **62**, 241–248
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275