Coupling ratio of electrogenic Na^+ –alanine cotransport in isolated rat hepatocytes

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(Received 3 December 1982/Accepted 21 December 1982)

Na^+ –alanine cotransport across the cell membrane in isolated rat hepatocytes was studied. Changes in the cell membrane potential associated with the transport of alanine interfere with determination of the Na^+ –alanine coupling ratio of the cotransport. With valinomycin present to ‘clamp’ the cell membrane potential, a coupling ratio of 1:1 for the Na^+ –alanine influx was obtained.

The transport of alanine across the liver cell membrane is an example of Na^+ –coupled electrogenic non-electrolyte transport (Kristensen, 1980), where the concentrative uptake of alanine is secondary to a coupled flow of Na^+ down the transmembrane Na^+ electrochemical gradient. According to this view the net uptake of alanine is sensitive to changes in the transmembrane Na^+ concentration difference as well as to changes in the cell membrane potential, and the coupling ratio between Na^+ and alanine is an essential aspect of the energetics of the cotransport (Aronson, 1981; Freel & Goldner, 1981). Operationally, the influx of Na^+ can be considered to consist of at least two components: (i) a ‘coupled’ Na^+ influx mediated by the Na^+ –alanine cotransport system and (ii) a ‘non-coupled’ Na^+ influx occurring by other pathways. The Na^+ –coupled alanine influx in hepatocytes is accompanied by transient depolarization (Folke & Paloheimo, 1975) followed by sustained hyperpolarization of the cell membrane (Dambach & Friedmann, 1974; Folke & Paloheimo, 1975). Any change in the cell membrane potential represents a change in the driving force, not only for the ‘coupled’ Na^+ –alanine influx, but also for the ‘non-coupled’ Na^+ influx to the extent that this latter is an electrogenic process. Therefore, via its effect on the cell membrane potential, the Na^+ –coupled alanine influx in hepatocytes might in principle influence the ‘non-coupled’ Na^+ influx. As also discussed by others (Philo & Eddy, 1978; Kimmich & Randles, 1980), this is a potential source of error in the determination of the coupling ratio. It is therefore desirable to measure this transport parameter under conditions where the cell membrane potential remains constant. Such conditions can be achieved with valinomycin, a K^+ –selective ionophore, which is expected to ‘clamp’ the cell membrane potential close to the K^+

equilibrium potential. In the present study, employing valinomycin-treated hepatocytes, we show that the Na^+ –alanine cotransport operates with a 1:1 coupling ratio.

Methods and materials

The methods used for preparation of isolated hepatocytes, incubations of the cells and measurements of the cellular uptake of Na^+ and alanine have been described in detail previously (Kristensen, 1980). Briefly, hepatocytes were isolated by collagenase perfusion of livers from 48 h-starved female Wistar rats (weight about 150 g). The cells were incubated in modified Krebs–Ringer bicarbonate buffers at pH 7.4 and 37°C. The buffer contained 2% (w/v) of defatted bovine albumin, and had the following composition (mM): Na^+ 135, K^+ 4.8, Ca^{2+} 2.4, Mg^{2+} 1.2, Cl^- 117.2, HCO_3^- 25, H_2PO_4^- 2.4, SO_4^{2-} 1.2. Amino-oxyacetate (2.5 mM) was present to obtain complete inhibition of the first step in the intracellular metabolism of alanine, thereby rendering alanine non-metabolizable during the measurements of alanine uptake and Na^+ uptake. At appropriate times during a 30 s period of incubation with ^{22}Na and/or ^{14}C –labelled alanine, the cellular uptake was terminated by centrifugation of 20 μl samples from the cell suspension, as follows. The samples were transferred to polypropylene tubes prepared with a bottom layer of 35 μl of 1 M- HClO_4 , a middle layer of 150 μl of silicone oil, and a top layer of 300 μl of incubation buffer containing 2.5 mM-ouabain and no radioisotopes. By this procedure a 16-fold dilution of the extracellular radioactivity concentration was obtained. The tubes were centrifuged immediately (within 1–2 s) in a Beckman Microfuge B. The starting time of the centrifuge was taken as the time

of termination of the cellular uptake. To obtain multiple samples within 30 s of incubation, a bank of four centrifuges was used. As described by Vinten *et al.* (1976), the centrifuges were connected to electronic timers and their starting times were printed out.

The HClO_4 cell precipitates and samples of the supernatants were counted for ^{22}Na radioactivity in a Packard Auto-Gamma spectrometer (model 5221) and for ^{14}C radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer (model 3375) at an appropriate setting for separation of ^{22}Na and ^{14}C radioactivity. The cellular uptake of $^{22}\text{Na}^+$ and ^{14}C -labelled alanine was expressed as the intracellular/extracellular distribution ratios. For this calculation the cellular water space and the amount of extracellular water trapped in the cell precipitate were determined in parallel experiments with $^3\text{H}_2\text{O}$ and hydroxy[^{14}C]methylinulin.

Materials

L-[U- ^{14}C]Alanine (sp. radioactivity about 200 Ci/mol, dissolved in 0.01 M-HCl) was from New England Nuclear Corp., Boston, MA, U.S.A., and other radioactive substances were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Valinomycin and other chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A., except collagenase (CLS II), which was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A., silicone oil AR200 (density 1.04 g/ml) from

Wacker Chemie, München, Germany, and ouabain from Fluka A.G., Buchs, Switzerland.

Results and discussion

The initial time courses of the cellular $^{22}\text{Na}^+$ uptake conformed to linearity when measured at an extracellular Na^+ concentration of 135 mM with or without 10 mM-alanine both in control cells and in cells treated with valinomycin (Fig. 1). As shown in Table 1, the 'non-coupled' Na^+ influx (alanine absent) was increased by valinomycin from 14.6 ± 0.3 to 16.3 ± 0.5 mmol/l of cell water per min. In the present experiments valinomycin could be expected to change the cell membrane potential from a resting value of about -30 mV (Folke, 1974; Dambach & Friedmann, 1974) to the K^+ equilibrium potential, which was about -90 mV $[(RT/F) \cdot \ln ([\text{K}^+]_o/[\text{K}^+]_i) = 26.7 \text{ mV} \cdot \ln (4.8/145)]$. The observation that such a change in the cell membrane potential increased the 'non-coupled' Na^+ influx significantly ($P < 0.05$, t test) can be taken as evidence that this component of the Na^+ influx is to some degree electrogenic, i.e. sensitive to changes in the cell membrane potential. On the other hand, the fact that valinomycin-induced increase in the 'non-coupled' Na^+ influx amounted to only 12% points to the possibility that a considerable fraction of the Na^+ influx in hepatocytes occurs by an electro-neutral pathway, e.g. Na^+-Cl^- cotransport, Na^+/Na^+ or Na^+/H^+ exchange. The presence of 10 mM-alanine in the extracellular medium stimulated the

Fig. 1. Time courses of basal and alanine-stimulated $^{22}\text{Na}^+$ uptake in the absence (a) and presence (b) of valinomycin. Isolated hepatocytes were suspended in a buffer containing 135 mM- Na^+ and 4.8 mM- K^+ and preincubated for 5 min with valinomycin (125 $\mu\text{g}/\text{ml}$) ($\blacktriangle$, $\triangle$) or with the solvent, ethanol (1%, w/w), as controls ($\bullet$, $\circ$). After preincubation, the uptake was initiated at zero time by addition of ^{22}Na , either without alanine ($\circ$, $\triangle$) or together with ^{14}C -labelled alanine at a final concentration of 10 mM ($\bullet$, $\blacktriangle$). At the times indicated, $^{22}\text{Na}^+$ accumulated in the cell water was determined as described in the Methods and materials section and expressed as the intracellular/extracellular distribution ratio. Each point represents the mean of four separate experiments with bars indicating $\pm$ S.E.M. when greater than the extension of the point. The lines of regression were calculated by the method of least squares on the assumption of intercept at the origin.

Table 1. *Effect of valinomycin on influxes of Na⁺ and alanine in isolated rat hepatocytes*

Na⁺ influxes and Na⁺-dependent alanine influxes were estimates from the slopes of the regression lines shown in Figs. 1 and 2. Each value is given as mean ± S.E.M. of the pooled data from four experiments. The apparent coupling ratio was calculated as the ratio between the alanine-dependent Na⁺ influx and the Na⁺-dependent alanine influx.

Cells	External [alanine] (mM)	Na ⁺ influx (mmol/l of cell water per min)	Na ⁺ -dependent alanine influx (mmol/l of cell water per min)	Apparent coupling ratio
Control	0	14.6 ± 0.3	—	—
Valinomycin	0	16.3 ± 0.5	—	—
Control	10	21.0 ± 0.5	8.1 ± 0.2	0.79
Valinomycin	10	26.7 ± 0.5	10.5 ± 0.2	0.99

Fig. 2. Time course of Na⁺-dependent ¹⁴C-labelled alanine uptake at an extracellular alanine concentration of 10 mM in the absence (a) and presence (b) of valinomycin

The uptake of ¹⁴C-labelled alanine in the presence of 135 mM-Na⁺ (●, ▲) was measured in the same incubations as shown and described in Fig. 1. In parallel experiments the alanine uptake was measured in hepatocytes suspended in Na⁺-free choline buffer (□). The lines of regression were calculated by the method of least squares on the assumption of intercept at the origin, and the difference between their slopes was taken as the Na⁺-dependent alanine influx.

influx of Na⁺ (Fig. 1 and Table 1), reflecting the operation of the Na⁺-alanine cotransport system. The alanine-induced increase in the Na⁺ influx was more pronounced in the valinomycin-treated cells, in accordance with the observation that valinomycin also stimulated the Na⁺-dependent alanine influx (Fig. 2 and Table 1). With valinomycin present, the Na⁺-alanine cotransport cannot be expected to influence the cell membrane potential to any appreciable degree, implying that all the fluxes needed for calculation of the coupling ratio are measured at a fixed value of the cell membrane potential. The data obtained under such conditions indicate that the Na⁺-alanine cotransport system in hepatocytes operates with a 1:1 coupling between Na⁺ and alanine (Table 1). In control cells some underestimation of the coupling ratio occurred, as

expected if the initial Na⁺-alanine influx had induced cell membrane depolarization and thereby a decrease in the electrogenic 'non-coupled' Na⁺ influx below the value measured in the absence of alanine. Compared with a previous attempt to determine the Na⁺-alanine coupling ratio (Kristensen, 1980), the present experiments represent an improved design, not only because of the introduction of valinomycin but also because the influxes of Na⁺ and alanine were measured simultaneously and were based on multiple samples.

The observed 1:1 coupling ratio agrees with the value favoured in several studies of glucose and amino acid influx across the intestinal and renal brush-border membrane (Curran *et al.*, 1967; Goldner *et al.*, 1969; Beck & Sactor, 1978; Hopfer & Groseclose, 1980) and studies of the Na⁺-

dependent amino acid uptake by Ehrlich cells (Schafer & Jacques, 1967; Eddy, 1968). More recently Kimmich & Randles (1980) obtained a Na^+ -glucose coupling ratio of 2:1 for the Na^+ -glucose influx across the brush-border membrane in chicken enterocytes under conditions where valinomycin and a high extracellular K^+ concentration were used to maintain the cell membrane potential constant and close to zero. It was suggested that in earlier studies the coupling ratio had been underestimated, owing to cell membrane depolarization induced by Na^+ -glucose transport under normal conditions. However, in another study of the Na^+ -glucose transport in rabbit intestinal and renal brush-border membrane vesicles, Hopfer & Groseclose (1980) circumvented the problem of cell membrane depolarization by using the equilibrium exchange technique. Under these conditions kinetic evidence in favour of a 1:1 coupling ratio was obtained. Shapiro & Kimmich (1980) have also employed their 'clamp' technique to determine the coupling ratio of Na^+ -valine influx across the chicken enterocyte brush-border membrane, and in that case a value of 1:1 was obtained. Thus the Na^+ -coupled amino acid influx in a variety of tissues seems to operate with a coupling ratio of 1 Na^+ :1 amino acid.

The present study implies that the ability of hepatocytes to concentrate alanine is a function of the first power of the Na^+ electrochemical gradient. Assuming that the Na^+ + K^+ -activated ATPase operates with a 3:1 molar ratio between Na^+ extrusion and ATP hydrolysis (Claret, 1979), the present study also implies that the energetic cost per mol of transmembrane influx of alanine in hepatocytes is $\frac{1}{3}$ mol of ATP.

We thank Betzy Hegner for skilful technical assistance and Rita Christiansen for secretarial assistance. Thanks

are due to Svend Christoffersen and Hans Valdal for construction of the electronic timing equipment. This work was supported by grants from The Novo Foundation and The Danish Foundation for Advancement of Medical Science.

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