Effect of ouabain on amino acid uptake by mouse ascites-tumour cells in the presence of nigericin

Elaine JOHNSON and A. Alan EDDY

Department of Biochemistry and Applied Molecular Biology, University of Manchester Institute of Science and Technology, P.O. Box 88, Manchester M60 1QD, U.K.

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1. Mouse ascites-tumour cells oxidizing lactate, in a modified Ringer solution, concentrated 2-aminoisobutyrate, L-methionine or 2-(methylamino)isobutyrate about 20-fold from a 0.4 mM solution in the presence of 2–3 μ g of nigericin/mg cellular dry wt. The ionophore increased cellular $[Na^+]$ to almost 100 mM when extracellular $[Na^+]$ was about 45 mM. 2. Either valinomycin or the two mitochondrial inhibitors oligomycin and antimycin acting together each markedly lowered the extent to which the tumour cells concentrated amino acid, from the above factor of about 20 to roughly 2-fold. 3. Ouabain (1 mM) had a similar effect, and further raised cellular $[Na^+]$. 4. The sodium pump appeared to be closely involved in amino acid uptake under these conditions.

Despite some uncertainties about the precise magnitude of the forces involved, there is substantial evidence that the Na^+ -dependent amino acid pumps of mouse ascites-tumour cells are driven by the electrochemical gradient of Na^+ ($\Delta\mu^{Na}$) acting across the plasma membrane (Gibb & Eddy, 1972; Henius & Laris, 1979; Johnstone, 1979; Heinz *et al.*, 1980; Eddy, 1982b; Hoffmann & Lambert, 1983). However, Heinz *et al.* (1981b) claim that $\Delta\mu^{Na}$ is much too small to maintain amino acid accumulation during aerobic energy metabolism in Ehrlich ascites-tumour cells kept in the presence of nigericin. Nigericin, which catalyses an electro-neutral exchange of Na^+ , K^+ and H^+ (Henderson, 1971; Reid *et al.*, 1974), appeared to lower $\Delta\mu^{Na}$ almost to zero in circumstances where the tumour cells concentrated 2-aminoisobutyrate extensively (Heinz *et al.*, 1981b). We reproduced some of the observations of Heinz *et al.* (1981b) with our tumour-cell line (Eddy & Johnson, 1983), and the present work is mainly concerned with two questions about this system. (1) Does the specific substrate of the Na^+ -dependent A system of amino acid transport, 2-(methylamino)isobutyrate, behave like the less specific 2-aminoisobutyrate

(Neville *et al.*, 1980; Ohsawa *et al.*, 1980; Eddy, 1982a)? (2) Does ouabain inhibit amino acid uptake in the presence of nigericin? Heinz *et al.* (1981b) found no inhibition, using 0.1 mM-ouabain for that purpose, a concentration which seems inadequate if an earlier estimate of the K_i value as 0.25 mM is relevant (Bittner & Heinz, 1963).

Materials and methods

These followed the general procedures described by Morville *et al.* (1973) and Philo & Eddy (1978). The mouse ascites-tumour cells (1 vol.) were first incubated for 30 min at 37°C in the standard Ringer solution (25 vol.). The latter contained 155 mM- Na^+ , 8 mM- K^+ , 131 mM- Cl^- , 16 mM-orthophosphate and 1.2 mM- $MgSO_4$. The cell preparation was collected by centrifugation, washed first in a Ringer solution of the same composition, and then in one containing 42 mM- Na^+ , 20 mM- K^+ , 101 mM-choline chloride, 16 mM-orthophosphate and 1.2 mM- $MgSO_4$. Portions (20 mg cellular dry wt.) were immediately transferred to a solution (10 ml) at 37°C containing 18 mM-DL-lactate, 42 mM- Na^+ , 20 mM- K^+ , 114 mM- Cl^- , 16 mM-orthophosphate, 101 mM-choline chloride and 1.2 mM- $MgSO_4$. The pH ranged from 7.0 to 7.2 on different occasions. Nigericin was added to the cell suspension 5 min later, either as an ethanolic solution or dissolved in dimethyl sulphoxide. The amounts of solvent used were innocuous in control

Abbreviations: $\Delta\mu^{Na}$, difference in the electrochemical potential of Na^+ in the extracellular solution and in the cytosol; $\Delta\psi$, difference in electrical potential across the plasma membrane, given a positive sign in the present paper.

tests. After a further 5 min at 37°C the appropriate amino acid was added to the cell suspension. Portions (1ml) were removed, layered over 1-bromododecane (0.5ml) and the tumour cells separated in an Eppendorf Microfuge at 10000g for 2 min. The supernatant solution was separated and portions were assayed for their ^{14}C content. The bromododecane was removed by aspiration and the compacted cellular pellet mixed with 0.6 ml of 0.01 M-HCl. After 30 min at 23°C the extract was separated from cellular debris and its ^{14}C content assayed. The cellular amino acid content was expressed in terms of either the cellular dry weight or its water content. The latter quantity, as well as the cellular contents of Na^+ and K^+ , were assayed separately, essentially as described by Morville *et al.* (1973). The sum of cellular $[\text{Na}^+] + \text{cellular } [\text{K}^+]$ was about 160 mM, whereas cellular water varied from about 2.9 to 3.6 $\mu\text{l}/\text{mg}$ cellular dry wt.

For certain purposes ouabain was either dissolved in the Ringer solution initially or added from a 1 M solution in dimethyl sulphoxide. Valinomycin, antimycin and oligomycin were added in solution in ethanol.

Cellular ATP

This was assayed by the method of Stanley & Williams (1969).

Chemicals

^3H -labelled inulin and the labelled amino acids were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Oligomycin, antimycin, nigericin and valinomycin were obtained from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K.

Results

Cellular contents of Na^+ and K^+ as a function of nigericin dose in the presence and absence of 1 mM-ouabain

Using a Ringer solution containing about 40 mM- Na^+ and 20 mM- K^+ , Heinz *et al.* (1981b) observed that, in the presence of 20 mM-L-lactate, 2 mM- Ca^{2+} , and 1% (w/v) bovine serum albumin to stabilize the cell suspension, a selected concentration of nigericin caused cellular $[\text{Na}^+]$ to increase during 30 min to about 100 mM while cellular $[\text{K}^+]$ fell correspondingly. The tumour cells meanwhile concentrated 1 mM-2-aminoisobutyrate about 12-fold, despite the outwardly directed concentration gradient with respect to $[\text{Na}^+]$. The plasma-membrane potential ($-\Delta\psi$) would thus need to be about -90 mV in order to account for the amino acid distribution in terms of $\Delta\mu^{\text{Na}}$ and the stoichiometric coefficient of 1 equiv. of Na^+ /mol of amino acid (Heinz *et al.*, 1981b).

Fig. 1 shows how our tumour-cell preparations behaved in a similar Ringer solution (Philo & Eddy, 1978) lacking albumin and Ca^{2+} and containing the indicated amount of nigericin. The cellular contents of Na^+ and K^+ were assayed 30 min after the addition of the ionophore in the presence or absence of 1 mM-ouabain. On the basis of the marked effect of ouabain illustrated in Fig. 1, we infer that the tendency of nigericin to cause Na^+ to accumulate in the tumour cells was opposed by the sodium pump.

Uptake of 2-aminoisobutyrate

The uptake of 2-aminoisobutyrate from a 0.4 mM solution was fairly constant in the period 20–30 min after addition of the amino acid to the Ringer solution containing lactate, 42 mM- Na^+ and 20 mM- K^+ (Fig. 3). Fig. 2 (●) shows how that constant uptake fell as the amount of nigericin in

Fig. 1. Increase in cellular $[\text{Na}^+]$ and decrease in cellular $[\text{K}^+]$ after exposure to increasing amounts of nigericin for 30 min in the presence or absence of 1 mM-ouabain

The cell suspension was prepared in a Ringer solution containing 10 mM-DL-lactate, 46 mM- Na^+ , 19 mM- K^+ , 0.4 mM-2-aminoisobutyrate and the indicated amount of nigericin. After 30 min at 37°C the tumour cells were collected, weighed and assayed for cellular water, Na^+ and K^+ (see the Materials and methods section). Observations made in three such experiments were combined to give mean values $\pm$ S.E.M. for the concentrations of Na^+ and K^+ , which are illustrated as a function of the nigericin dose ($\mu\text{g}/\text{mg}$ cellular dry wt.). Ouabain present: $[\text{Na}^+]$, ○; $[\text{K}^+]$, △. Ouabain absent: $[\text{Na}^+]$, ●; $[\text{K}^+]$, ▲.

Fig. 2. Effects of 1 mM-ouabain and of different amounts of nigericin on uptake of 2-aminoisobutyrate at 30 min, measured relative to a control lacking nigericin: influence of lactate or glucose provision

The tumour cells absorbed 2-amino[^{14}C]isobutyrate for 30 min from a 0.4 mM solution in conditions similar to those used for Fig. 1, except that the carbon substrate varied. Series 1 contained 18 mM-DL-lactate, either with 1 mM-ouabain (○) or without it (●). Uptake with nigericin present is expressed as a percentage of a control lacking nigericin and ouabain. Series 2 contained 10 mM-glucose (▲), as did the corresponding control. No carbon substrate was added to series 3 (■), uptake in the presence of nigericin being expressed relative to a control without it. Each point is the mean $\pm$ S.E.M. for three to seven observations made in the course of 30 expts. Typical values of the amino acid accumulation ratio are illustrated in Table 1.

the system was increased. The scatter of the observations necessitated the averaging of the results of several consecutive experiments. Heinz *et al.* (1981b) found that omission of lactate decreased amino acid accumulation, whereas its replacement by glucose increased amino acid accumulation. Similar trends are apparent in Fig. 2

Fig. 3. Time course of the uptake of 2-aminoisobutyrate in the presence of nigericin as a function of ouabain concentration

Uptake of the amino acid occurred from a 0.4 mM solution at 37°C in the presence of ouabain at 1 mM (□), 0.3 mM (Δ), 0.1 mM (○) or in its absence (●). The Ringer solution contained 42 mM- Na^+ , 20 mM- K^+ , 10 mM-DL-lactate and 2.6 μg of nigericin/mg cellular dry wt.

when lactate was omitted (■) or replaced by glucose (▲).

Effect of ouabain on amino acid uptake

Addition of 1 mM-ouabain to the system inhibited the uptake of 2-aminoisobutyrate by about 50% in the absence of nigericin, an effect that increased markedly in the presence of relatively small amounts of nigericin (Fig. 2, ○ and ●). The result of varying the ouabain concentration in the range up to 1 mM is illustrated in Fig. 3. The mean value of two determinations of K_i for ouabain, acting as a non-competitive inhibitor, was about 0.24 mM (type IIa kinetics; Dixon & Webb, 1964). An earlier comparable study with mouse Ehrlich ascites cells gave 0.25 mM (Bittner & Heinz, 1963). The mean cellular ATP content ($\pm$ S.E.M.) in three cell preparations kept under the conditions illustrated in Fig. 2 was 8.3 ± 1.2 nmol/mg in the absence of ouabain and nigericin. The ATP content decreased by $28 \pm 7\%$ in the presence of nigericin (1.1 μg/mg) and by $15 \pm 13\%$ in the presence of 1 mM-ouabain as well. Thus ouabain was not acting simply as an inhibitor of ATP formation.

Effect of albumin

As Heinz *et al.* (1981b) did most of their work with lactate in Ringer solutions containing 1% (w/v) albumin, the question arose whether the presence of the protein influenced the actions of nigericin on the system. Grinstein *et al.* (1983) have indeed used albumin to nullify the effect of a previous addition of nigericin to suspensions of human peripheral blood cells. The albumin plausibly may bind a fraction of the nigericin outside the cells. Fig. 4 shows that the addition of albumin to the Ringer solution containing 48 mM- Na^+ resulted in a larger uptake of 2-aminoisobutyrate at a given concentration of nigericin. Ouabain inhibited 2-aminoisobutyrate uptake irrespective of the presence of albumin (Fig. 2; Fig. 4, Δ). The experiments illustrated in Fig. 4 were done about 1 year after those shown in Figs. 1, 2 and 3. Some increase

Fig. 4. Uptake of 2-aminoisobutyrate during 30 min in the presence of various amounts of nigericin, with and without 1% (w/v) albumin present

Amino acid uptake from a 0.4 mM solution proceeded as illustrated in Fig. 3 in the presence (O) or absence (●) of albumin. The Ringer solution contained 18 mM-DL-lactate and 48 mM- Na^+ to which the albumin contributed a further 4 mM. $[\text{K}^+]$ was 18 mM. The cellular content of water was assumed not to change in the presence of albumin. Observations made on three to six occasions were combined to give a mean value $\pm$ S.E.M. for the ratio [cellular 2-aminoisobutyrate]/[extracellular 2-aminoisobutyrate] ($[\text{A}]_i/[\text{A}]_o$). The effect of 2 mM-ouabain on the system containing albumin is also shown (Δ).

in the magnitude of the effect of a given dose of nigericin is apparent, relative to the trends shown in Fig. 2.

In three series of assays, increasing the nigericin dose progressively lowered the cellular ATP content relative to controls lacking nigericin, by $28 \pm 7\%$, $48 \pm 9\%$ and $85 \pm 7\%$ (S.E.M.) respectively, with 1.1, 1.8 and 3 μg of nigericin/mg. It may be relevant that relatively large amounts of nigericin can inhibit oxidative phosphorylation (Henderson, 1971).

Other amino acids

A series of studies with 2-aminoisobutyrate, 2-(methylamino)isobutyrate and L-methionine is summarized in Table 1. Each amino acid was concentrated extensively in the presence of nigericin, the process being inhibited by ouabain. The amino acid accumulation ratio for 2-aminoisobutyrate did not change greatly when its concentration was increased from 0.1 mM to 2 mM (series 1, Table 1).

Effect of mitochondrial inhibitors

These were used at the concentrations that Philo & Eddy (1978) employed to inhibit mitochondrial energy metabolism. The presence of antimycin and oligomycin, either with or without 2,4-dinitrophenol, almost stopped the concentration of 2-aminoisobutyrate (series 4, Table 1). That the tumour cells probably remained intact is shown by the fact that in four similar further assays the mean volume ($\pm$ S.E.M.) of cellular water from which ^3H -labelled inulin was excluded was $4.23 \pm 0.28 \mu\text{l}/\text{mg}$ after 40 min in the presence of nigericin, oligomycin, antimycin and 2,4-dinitrophenol, which is to be compared with the value of 3.03 ± 0.13 observed in parallel assays with the same amount of nigericin (1.5 $\mu\text{g}/\text{ml}$) but lacking the other compounds. Reference to Table 1 (series 4) shows further that a transient accumulation of amino acid was apparent 5 min after its addition to the system, which by then had been exposed to nigericin for 10 min and to the other metabolic inhibitors for 15 min. A related phenomenon was noted by Heinz *et al.* (1981b). In other experiments we added either valinomycin or ouabain to cell suspensions that had accumulated 2-aminoisobutyrate for 30 min (series 5, Table 1). The former compound acting in the presence of nigericin would stop oxidative phosphorylation (Henderson, 1971). Table 1 shows that the expected rapid efflux of amino acid occurred relative to the controls.

Discussion

The circumstances described by Heinz *et al.* (1981b) are an exacting test of the co-substrate

Table 1. Accumulation of 2-(methylamino)isobutyrate, L-methionine or 2-aminoisobutyrate by the mouse ascites-tumour cells in the presence of nigericin, and the effects of ouabain or of mitochondrial inhibitors on the process

The amino acid was absorbed from a solution of the indicated concentration in a Ringer solution containing 42 mM- Na^+ , 20 mM- K^+ , 18 mM-DL-lactate, 101 mM-choline chloride and the other compounds listed below. The nigericin present varied from about 2 to 3 $\mu\text{g}/\text{ml}$ cellular dry wt. in the five series of assays. In general the tumour-cell suspension was kept at 37°C for 5 min in either the presence or the absence of ouabain, nigericin was added, and 5 min later the appropriate amino acid labelled with ^{14}C . Cellular ^{14}C was assayed at the indicated times after addition of ^{14}C . Except where indicated ($\pm$ S.E.M.), the ratio of the cellular to extracellular amino acid concentrations ($[A]_i/[A]_o$) is the mean of two similar values obtained on separate occasions after addition of ^{14}C . In series 5 either valinomycin or ouabain was added after the tumour cells had accumulated 2-amino[^{14}C]isobutyrate for 30 min.

	at $t =$	Amino acid accumulation ratio	
		20 min	30
Series 1			
0.1 mM-2-aminoisobutyrate		25.5	24.2
0.4 mM-2-aminoisobutyrate		15.6	23.8
2.0 mM-2-aminoisobutyrate		9.5	13.9
Series 2			
0.4 mM-2-(methylamino)isobutyrate		19.3 $\pm$ 4.0(4)	21.2 $\pm$ 4.4(4)
0.4 mM-2-(methylamino)isobutyrate + 2 mM-ouabain		2.3 $\pm$ 0.2(4)	2.1 $\pm$ 0.1(4)
Series 3			
0.4 mM-L-methionine		21.0	19.7
0.4 mM-L-methionine + 2 mM-ouabain		2.3	2.4
Series 4	at $t =$	20 min	30 min
Expt. 1			
0.4 mM-2-aminoisobutyrate	9.9	21.8	26.4
0.4 mM-2-aminoisobutyrate + antimycin (0.5 $\mu\text{g}/\text{mg}$) + oligomycin (12 ng/mg)	3.9	2.6	2.0
Expts. 2 and 3			
0.2 mM-2-aminoisobutyrate	10.7	14.8	14.1
0.2 mM-2-aminoisobutyrate + antimycin (2.7 $\mu\text{g}/\text{mg}$) + oligomycin (14 ng/mg) + 60 μM -2,4-dinitrophenol	2.8	1.7	1.5
Series 5	at $t =$	30 min	60 min
Expt. 1			
0.4 mM-2-aminoisobutyrate	17.7	21.5	12.4
0.4 mM-2-aminoisobutyrate, + valinomycin (1.3 $\mu\text{g}/\text{mg}$) at $t = 30$ min		3.3	1.5
0.4 mM-2-aminoisobutyrate, + 1.6 mM-ouabain at $t = 30$ min		8.7	2.7
Expt. 2			
0.4 mM-2-aminoisobutyrate	8.4	8.3	7.6
0.4 mM-2-aminoisobutyrate, + valinomycin (1.4 $\mu\text{g}/\text{mg}$) at $t = 30$ min		2.0	1.6
0.4 mM-2-aminoisobutyrate, + 2 mM-ouabain at $t = 30$ min		4.3	2.0

hypothesis in that cellular $[Na^+]_i$ ($[Na^+]_i$) stays near 90 mM for at least 30 min in the presence of lactate when extracellular $[Na^+]_o$ ($[Na^+]_o$) is 40–50 mM (Fig. 1). L-Methionine, 2-aminoisobutyrate and 2-(methylamino)isobutyrate were each concentrated about 15–25-fold under these conditions (Table 1), probably through the Na^+ -dependent A transport system (Ohsawa *et al.*, 1980). This behaviour is only consistent with the gradient hypothesis, in which the tumour cells absorb proportionately 1 equiv. each of Na^+ and the amino acid, if $\Delta\psi$ is about 90 mV (Heinz *et al.*, 1981b). Transient membrane potentials of that magnitude have indeed been observed during glycolysis (Philo & Eddy, 1978; Heinz *et al.*, 1981a; Bashford & Pasternak, 1984), but further work is needed to show whether they are produced during respiration of lactate. A possible mechanism of hyperpolarization is suggested by earlier studies, in the absence of nigericin, with cell preparations in which initially $[Na^+]_i > [Na^+]_o$ and the sodium pump lowered $[Na^+]_i$, considerably within about 15 min. During that interval ouabain and valinomycin each inhibited amino acid absorption and lowered $\Delta\psi$ (Gibb & Eddy, 1972; Laris *et al.*, 1978; Philo & Eddy, 1978; Heinz *et al.*, 1980). The sodium pump initially hyperpolarized the various tumour cells up to 63 mV (Laris *et al.*, 1978) or 100 mV (Philo & Eddy, 1978), and $\Delta\mu^{Na}$ was probably sufficiently large to drive the amino acid pumps (Johnstone, 1979; Heinz *et al.*, 1980; Eddy, 1982b).

We suggest that both amino acid absorption and $\Delta\psi$ were influenced by similar factors when $[Na^+]_i$ was near 100 mM in the persistent steady state that the tumour cells maintained in the presence of nigericin. Thus the effect of ouabain on amino acid uptake (Table 1, Fig. 3) and its effect on the magnitude of $[Na^+]_i$ (Fig. 1) imply that the action of the sodium pump assists Na^+ -dependent solute absorption. Interactions between ouabain, monensin and amino acid uptake were noted by Smith & Austic (1980), and between nigericin and the effects of ouabain on $\Delta\psi$ by Bashford & Pasternak (1984). We liken the proposed hyperpolarization of the plasma membrane by the sodium pump in the presence of nigericin to the mitochondrial hyperpolarization that nigericin may cause (Henderson, 1971). The possibility that nigericin may affect $\Delta\psi$ at the plasma membrane by changing the intracellular pH needs to be borne in mind.

Heinz *et al.* (1981b) also considered the possibility of membrane hyperpolarization assisting amino acid uptake during respiration-driven ion pumping in the presence of nigericin, but rejected it for two main reasons. (1) Neither 0.1 mM-ouabain nor 50 μ M-vanadate decreased the relatively small amino acid uptake occurring in the absence of lactate (Heinz *et al.*, 1981b). The K_i for

ouabain is, however, about 0.25 mM (Bittner & Heinz, 1963). We accordingly question whether Heinz *et al.* (1981b) used enough ouabain to inhibit the sodium pump decisively (cf. Morville *et al.*, 1973). (2) Unlike glucose, lactate failed to hyperpolarize the tumour cells in an assay based on a voltage-sensitive carbocyanine dye (Heinz *et al.*, 1981b). The assay system employed inhibitors of oxidative phosphorylation to stop spurious signals coming from the mitochondria (Philo & Eddy, 1978). Table 1 shows that similar metabolic inhibitors lowered the uptake of 2-aminoisobutyrate in the presence of lactate, presumably by hindering energy metabolism. Clearly, the need (a) to maintain oxidative phosphorylation during the catabolism of endogenous substrates, or of lactate itself, conflicts with the need (b) to eliminate artifactual signals arising in the same organelle, and effectively rules out the use of the carbocyanine dye in this specific application. Bashford & Pasternak (1984) use an oxonol dye to assay $\Delta\psi$, and propose that the dye is excluded from the mitochondrial compartment of the tumour cells (cf. Montecucco *et al.*, 1979). We have studied the fluorescence of the oxonol during energy metabolism in the presence of lactate and nigericin, and have found (E. Johnson & A. A. Eddy, unpublished work) that $\Delta\psi$, assayed by that means, is considerably larger than Heinz *et al.* (1981b) inferred from the behaviour of the carbocyanine. We note nevertheless that the latter gives satisfactory results when ATP is generated by glycolysis (Johnstone *et al.*, 1982).

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