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Short-term stimulation of Na^+-dependent amino acid transport by dibutyryl cyclic AMP in hepatocytes

Characteristics and partial mechanism

S. Kelly MOULE, Norah M. BRADFORD and John D. McGIVAN
Department of Biochemistry, Medical School, University Walk, Bristol BS8 1TD, U.K.

1. The short-term protein-synthesis-independent stimulation of alanine transport in hepatocytes was further investigated. 2. Cyclic AMP increased the $V_{max.}$ of alanine transport. 3. Amino acid transport via systems A, ASC and N was stimulated. 4. A good correlation was found between the initial rate of transport and the cell membrane potential as calculated from the distribution of Cl^-. 5. Cyclic AMP increased the rate of alanine transport, stimulated Na^+/K^+ ATPase (Na^+/K^+-transporting ATPase) activity and caused membrane hyperpolarization. The time courses and cyclic AMP dose-dependencies of all three effects were similar. 6. Ouabain abolished the effect of cyclic AMP on Cl^- distribution and on transport of alanine. 7. The effect of cyclic AMP on alanine transport and Cl^- distribution was mimicked by the antibiotic nigericin; the effect of nigericin was also abolished by ouabain. 8. It is concluded that the effect of cyclic AMP on transport is mediated via membrane hyperpolarization. It is suggested that the primary action of cyclic AMP is to increase the activity of an electroneutral Na^+/K^+-exchange system in the liver cell plasma membrane, thus hyperpolarizing the membrane by stimulating the electrogenic Na^+/K^+ ATPase.

INTRODUCTION

The Na^+-dependent transport of neutral amino acids into isolated hepatocytes is subject to both short-term and long-term regulation. Transport via system A is stimulated by insulin, corticosteroids and catecholamines. These effects occur after a prolonged lag period and are sensitive to inhibitors of DNA and protein synthesis. System A activity is also subject to adaptive control. The hormonal and adaptive regulation of this transport system is considered to be due in part to synthesis and insertion of new carrier molecules into the cell membrane. This long-term regulation of amino acid transport in hepatocytes has been the subject of several reviews (Kilberg, 1982; Shotwell *et al.*, 1983; Kilberg *et al.*, 1985; Kilberg, 1986).

Edmondson & Lumeng (1980) analysed the stimulation of alanine transport by glucagon in isolated hepatocytes. Glucagon exerted a transient short-term stimulation, succeeded by a longer-term increase in transport, which was abolished by cycloheximide. On the basis of a comparison of the effects of glucagon with those of valinomycin, it was suggested that the short-term activation of alanine transport by glucagon was due to hyperpolarization of the cell membrane by this hormone, an effect which had been shown to occur in perfused liver (Friedmann & Dambach, 1973; Petersen, 1974). It has also been shown that alanine transport in hepatocytes is stimulated by cyclic AMP, and this stimulation is independent of protein synthesis (McGivan *et al.*, 1981). The exact mechanism by which glucagon and cyclic AMP exert their effect is still unclear, since no satisfactory method of estimating the membrane potential in isolated hepatocytes has been devised.

In this paper, the effect of cyclic AMP on amino acid transport in isolated hepatocytes is characterized in more detail. Evidence is presented which is consistent with stimulation of Na^+-dependent transport via a change in membrane potential, and a partial mechanism for the hyperpolarization of the cell membrane by cyclic AMP is proposed.

MATERIALS AND METHODS

Hepatocytes were isolated from the livers of 24 h-starved male Wistar rats as described previously (Bradford *et al.*, 1985) and were suspended in Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 2% (w/v) dialysed bovine albumin and equilibrated with O_2/CO_2 (19:1). The medium also routinely contained 1 mM-amino-oxyacetate to inhibit the metabolism of alanine (Edmondson *et al.*, 1977; Joseph *et al.*, 1978). The cells were incubated at 37 °C in a rotary shaker. The final cell concentration after all additions was approx. 5 mg of protein/ml.

Alanine transport was measured by using [^{14}C]alanine, together with [^{3}H]inulin as a marker of the extracellular space. Transport was terminated by centrifugation of the cell suspension through silicone oil into $HClO_4$ as described previously (Joseph *et al.*, 1978). The initial rate of transport of glycine, serine and glutamine was determined similarly. In the presence of amino-oxyacetate the rate of metabolism of all these amino acids was negligible over the time intervals used.

The distribution of $^{36}Cl^-$ was measured as described previously (Bradford *et al.*, 1985) in the presence of [^{3}H]inulin as a marker of the extracellular space. Parallel experiments were performed in which cells were incubated with ^3H_2O together with inulin[^{14}C]carboxylic acid to measure the intracellular volume. For each determination, quadruplicate samples were taken and the Cl^- distribution was calculated as the mean of these values.

Abbreviation used: Na^+/K^+ ATPase, Na^+/K^+-transporting adenosine triphosphatase (EC 3.6.1.37).

Na^+/K^+ ATPase activity was measured as the uptake of $^{86}Rb^+$ that was inhibited by 1 mM-ouabain (Ihlenfeldt, 1981). In this case the extracellular space was determined by using [^{3}H]inulin in parallel experiments. Details of the experimental procedure have been published previously (Bradford *et al.*, 1985). Protein was measured by a biuret method (Gornall *et al.*, 1949), with bovine albumin as a standard.

Radioisotopes were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Collagenase was purchased from Worthington Biochemicals.

RESULTS

Short-term effects of dibutyryl cyclic AMP, ouabain and uncoupling agents on Na^+-dependent amino acid transport

Dibutyryl cyclic AMP has been shown to increase the initial rate of alanine transport via a protein-synthesis-independent mechanism which occurs over a period of minutes (McGivan *et al.*, 1981). In common with other Na^+-dependent transport processes, alanine transport is inhibited by ouabain, which inhibits Na^+/K^+ ATPase activity. Uncoupling agents, which lower the cell ATP concentration, also inhibit transport (Joseph *et al.*, 1978). In preliminary experiments, it was shown that the uptake of alanine was approximately linear with time for the first 2 min, but the rate decreased markedly after this period in the presence of inhibitors. The initial rate of alanine transport in subsequent experiments was therefore calculated as uptake over the first 2 min.

The effects of cyclic AMP, ouabain and uncoupling agents on alanine transport are mediated via a change in the $V_{max.}$ of the transport process (Fig. 1). The $V_{max.}$ values (nmol/min per mg) obtained were as follows: control, 21.2; cyclic AMP, 26.6; carbonyl cyanide *m*-chlorophenylhydrazone, 14.3; ouabain, 13.3. The apparent K_m for alanine was 4.5 mM in each case, and this is consistent with previous reports (Joseph *et al.*, 1978; Sips *et al.*, 1980*a*).

In isolated hepatocytes, three Na^+-dependent systems (A, ASC and N) have been identified for the transport of neutral amino acids (see Kilberg, 1982). In addition to its stimulation of alanine transport, cyclic AMP stimulated the transport of glycine and serine, which are also substrates for system A. This effect was similar in magnitude to that found for alanine transport (results not shown). The transport of glutamine in hepatocytes occurs via System N. The initial rate of transport of glutamine (0.5 mM) was 3.98 ± 0.32 nmol/min per mg, and was increased to 4.32 ± 0.33 nmol/min per mg in the presence of 0.2 mM-cyclic AMP ($n = 7$ cell preparations; $P < 0.001$, by a paired t test). Transport via system ASC can be assessed by measuring the Na^+-dependent component of alanine transport (at 0.1 mM-alanine) in the presence of 25 mM-*N*-methyl-α-aminoisobutyrate, a specific inhibitor of system A. Table 1 shows that alanine transport via system ASC was also stimulated by cyclic AMP.

Correlation between cell membrane potential and initial rate of alanine transport

The Na^+-dependent electrogenic transport of neutral amino acids across the cell membrane would be expected to respond kinetically to changes in the cell membrane potential (Geck & Heinz, 1976). However, the membrane potential in isolated hepatocytes has not been satisfactorily determined by direct electrophysiological methods. The potential cannot be estimated from the distribution of lipid-soluble cations, since these are accumulated by the large number of mitochondria in these cells; lipid-soluble cationic dyes are unsatisfactory indicators of the liver cell membrane potential for the same reason. In principle, the membrane potential can be determined from the distribution of permeant anions. Although thiocyanate has been used for this purpose (Edmondson *et al.*, 1986), other workers have concluded that corrections must be made for the binding of thiocyanate to intracellular constituents (Hoek *et al.*, 1980). In our hands, thiocyanate distribution in isolated hepatocytes does not coincide with the cell membrane potential previously measured in perfused liver.

Cl^- was found to cross the liver cell membrane relatively rapidly (Fig. 2), and distribution of Cl^- was unaffected by the addition of 0.1 μM-4,4′-diisothio-

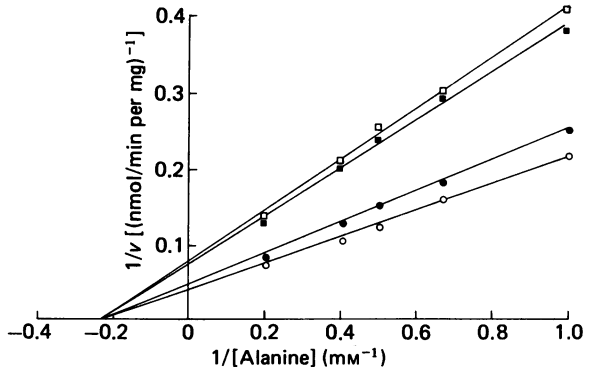

Fig. 1. Double-reciprocal plot of the initial rate of alanine transport as a function of alanine concentration in the presence of various effectors

Cells were preincubated for 30 min with (●) no further additions, (○) dibutyryl cyclic AMP (0.1 mM), (□) carbonyl cyanide *m*-chlorophenylhydrazone (50 μM), or (■) ouabain (1 mM) as indicated, before measurement of the initial rate over a period of 2 min.

Table 1. Effect of *N*-methylaminoisobutyrate on the transport of 0.1 mM-alanine in the presence and absence of dibutyryl cyclic AMP

Cells were preincubated for 30 min with or without 0.2 mM-cyclic AMP, and the transport of alanine (0.1 mM) in the presence or absence of 25 mM-*N*-methyl-α-aminoisobutyrate (MeAIB) was measured over a period of 2 min. The results are means ± S.E.M. of values derived from three separate cell preparations. Significance was assessed by Student's t test. In control experiments, the rate in the absence of Na^+ was < 0.04 nmol/min per mg and was not affected by cyclic AMP. $*P < 0.01$, $**P < 0.001$ versus control; $\dagger P < 0.05$ versus MeAIB alone.

Additions	Initial rate of alanine transport (nmol/min per mg)
None	0.54 ± 0.025
Cyclic AMP	0.76 ± 0.015*
MeAIB	0.29 ± 0.012**
MeAIB + cyclic AMP	0.37 ± 0.023*†

cyanatostilbene-2,2'-disulphonate, 0.1 mM-4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate or 50 μM-furosemide (results not shown), inhibitors of Cl^- transport systems in some cells. Bumetanide, an inhibitor of the ($Na^+ + K^+ + 2Cl^-$) transporter, has been shown to be without effect in hepatocytes (Bakker-Grunwald, 1983). The membrane potential calculated from Cl^- distribution in hepatocytes has been found to correspond closely to the potential measured directly with microelectrodes under a range of similar conditions in perfused liver (Bradford *et al.*, 1985). Accordingly, the relationship between alanine transport and Cl^- distribution as a putative indicator of cell membrane potential was determined.

Fig. 3 shows the relationship between the initial rate of alanine transport at 0.5 mM-alanine and the steady-state Cl^- distribution in hepatocytes. The results shown were obtained from experiments on eight separate cell preparations; cells were incubated in the presence of ouabain, glucagon, dibutyryl cyclic AMP or carbonyl cyanide *m*-chlorophenylhydrazone. In some experiments the cells were incubated in a medium containing high concentrations of KCl. Under all conditions there was an inverse correlation between the Cl^- distribution (expressed as the ratio of internal to external concentrations) and the initial rate of transport. Glucagon and cyclic AMP decreased the Cl^- distribution ratio and stimulated transport, whereas carbonyl cyanide *m*-chlorophenylhydrazone, ouabain and medium containing a high KCl concentration increased the distribution ratio of Cl^- and decreased the rate of transport. In the experiments with a high K^+ concentration, some of the decrease in rate must be attributed to the lower Na^+ concentration present.

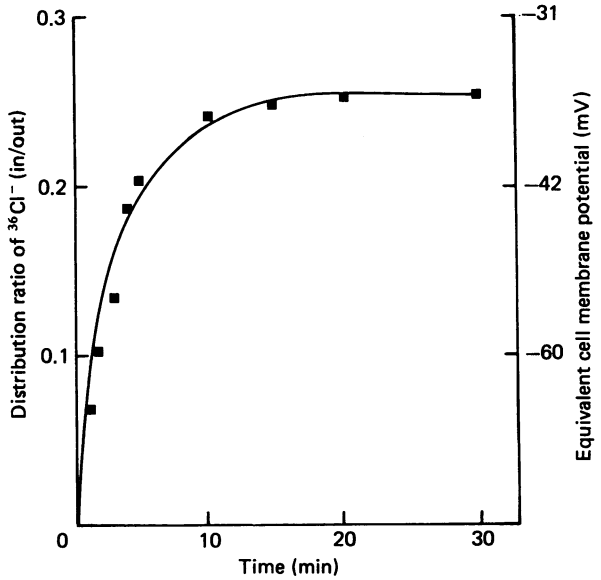

Fig. 2. Time course of $^{36}Cl^-$ distribution across the hepatocyte plasma membrane

Cells were preincubated for 15 min, then $Na^{36}Cl$ (0.4 μCi/ml final concn.) and [^{3}H]inulin (2 μCi/ml) were added. Samples of the cell suspension were centrifuged after the times shown for determination of Cl^- distribution. The internal volume of the cells was determined in parallel experiments.

Fig. 3 also shows the apparent cell membrane potential calculated from Cl^- distribution. Changing the potential from −35mV to −25mV decreased the rate of transport by a factor of 2, whereas altering the potential from −35 mV to −50 mV increased the rate by approx. 30%. No conditions were found where an agent that influenced Cl^- distribution failed to alter the initial rate of transport, or vice versa.

These results may be interpreted to indicate that the initial rate of transport of alanine is a function of the cell membrane potential and that the stimulation of transport by cyclic AMP is brought about by a hyperpolarization of the hepatocyte plasma membrane, as previously suggested for the short-term stimulation of transport by glucagon (Edmondson & Lumeng, 1980).

Mechanism of cell membrane hyperpolarization by cyclic AMP

Cyclic AMP has been shown to increase the expressed Na^+/K^+ ATPase activity in hepatocytes (Ihlenfeldt,

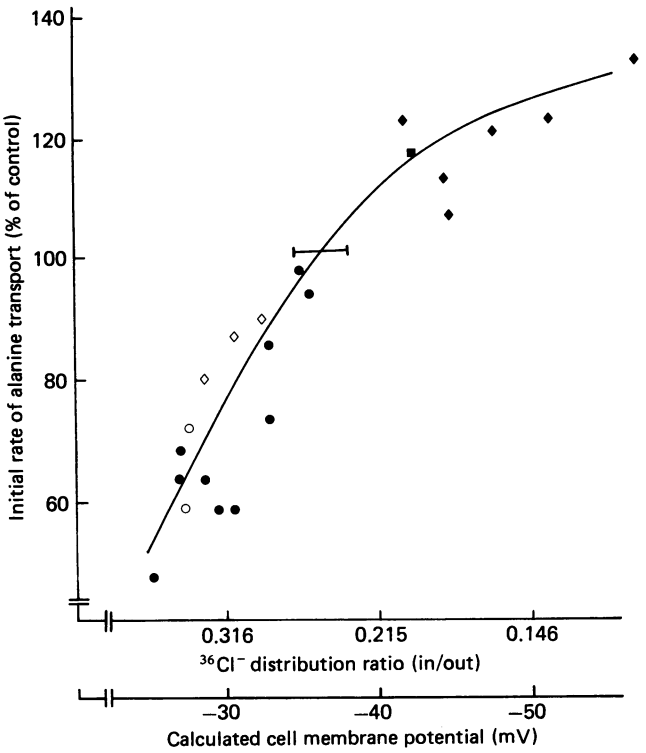

Fig. 3. Relationship between the initial rate of alanine transport and Cl^- distribution in hepatocytes

Cells were incubated with various effectors, and the Cl^- distribution and initial rate of alanine transport (at 0.5 mM-alanine) were determined as described in the Materials and methods section. The results shown were taken from experiments on eight separate cell preparations, and the rate in each determination was taken as a percentage of the control rate for that particular cell preparation. The mean control rate of alanine transport was 2.00±0.18 nmol/min per mg (mean±S.E.M., $n = 8$). The membrane potential calculated from the Cl^- distribution is also shown. The effectors used were: ◆, dibutyryl cyclic AMP (0–0.2 mM); ●, carbonyl cyanide *m*-chlorophenylhydrazone (0–50 μM); ○, ouabain (1 mM); ■, glucagon (300 nM); ◇, KCl (15–50 mM) replacing an equal concentration of NaCl in the incubation medium.

1981). Since the Na^+/K^+ ATPase is electrogenic, increased activity of this enzyme would tend to cause hyperpolarization of the cell membrane. If the cyclic AMP-mediated changes in membrane potential were a consequence of increased Na^+/K^+ ATPase activity, the time courses of the observed changes in ATPase activity and Cl^- distribution should be similar.

Na^+/K^+ ATPase activity was measured by following the ouabain-sensitive uptake of $^{86}Rb^+$ ions into isolated hepatocytes, as described by Ihlenfeldt (1981). Fig. 4 shows that time courses of the increase in alanine transport, change in Cl^- distribution and stimulation of Na^+/K^+ ATPase activity were similar, with a half-maximal effect occurring after 10 min in each case. The three parameters shown in Fig. 4 also exhibited closely similar dose-dependencies on cyclic AMP, with half-maximum effects at 10–30 μM (results not shown). Inhibition of the Na^+/K^+ ATPase by ouabain has been previously shown to abolish the effect of cyclic AMP on Cl^- distribution (Bradford *et al.*, 1985). These results are consistent with the postulate that the effect of cyclic AMP on membrane potential, and hence on transport, is mediated via an increase in Na^+/K^+ ATPase activity.

The specificity of the short-term stimulation of alanine transport was investigated. The hormones glucagon, phenylephrine and vasopressin all exert short-term effects on liver metabolism which are independent of protein synthesis. Table 2 shows the effects of these hormones, at concentrations which are known to affect metabolism in hepatocytes, on alanine transport, Cl^- distribution and Na^+/K^+ ATPase activity. The effect of glucagon was qualitatively similar to that of cyclic AMP on all three parameters. The Ca^{2+}-dependent hormones phenylephrine and vasopressin did not stimulate alanine transport, and had no effect on either Cl^- distribution or on Na^+/K^+ ATPase activity under the conditions used.

Na^+/K^+ ATPase activity in cells is considered to be regulated mainly by the intracellular concentration of Na^+ ions. The antibiotic nigericin catalyses an electroneutral 1:1 exchange of Na^+ for either K^+ or H^+ across membranes, and should thus increase Na^+/K^+ ATPase activity and consequently cause membrane hyperpolarization. Nigericin was found to increase the membrane potential from −35 mV to −41 mV, as calculated from Cl^- distribution. Fig. 5 shows that nigericin produced a dose-dependent stimulation of alanine transport, which was maximal at 10 nM; at higher nigericin concentrations the effect decreased, probably owing to large net changes in ion gradients and to progressive interference of the antibiotic with mitochondrial function.

Table 3 shows the characteristics of the stimulation of alanine transport by cyclic AMP and by nigericin. The stimulation of transport by nigericin was quantitatively similar to that produced by cyclic AMP. Inhibition of the Na^+/K^+ ATPase by ouabain partially inhibited the control rate of transport and abolished the stimulation of transport by both effectors. In the presence of a saturating concentration of cyclic AMP, nigericin

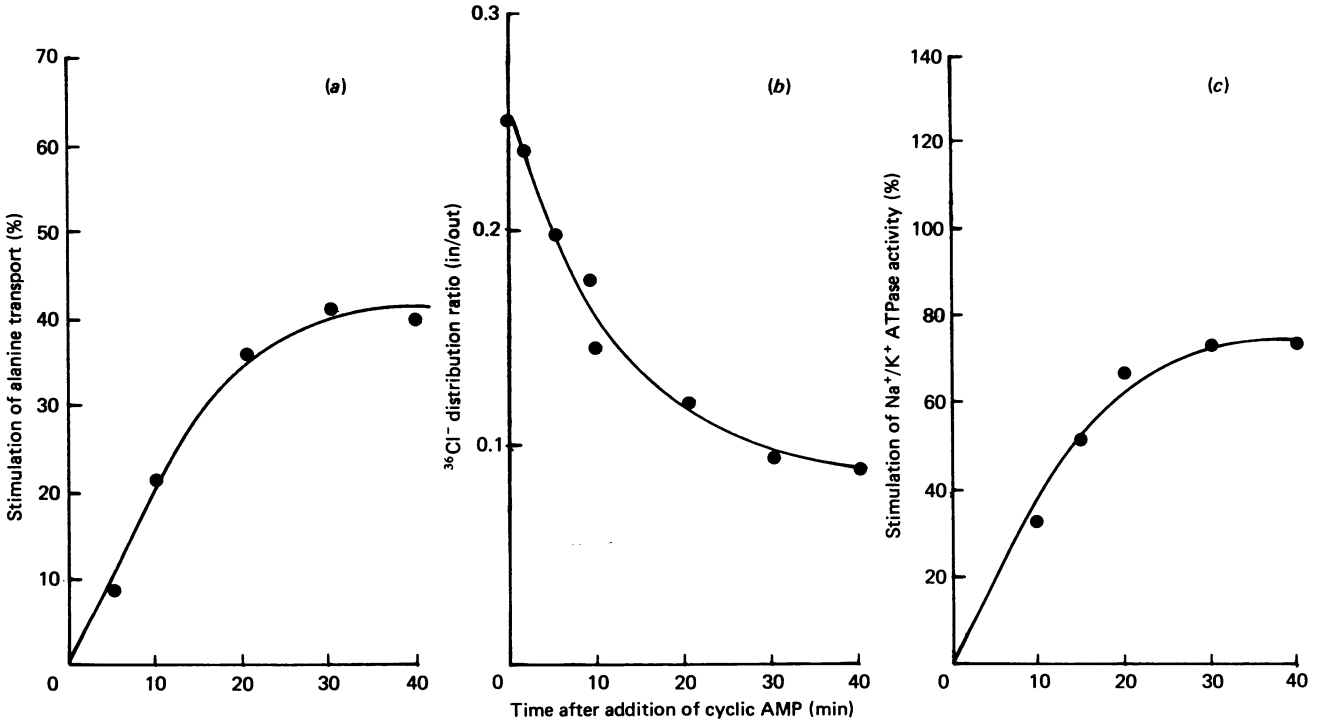

Fig. 4. Time course of the effect of cyclic AMP on (*a*) alanine transport, (*b*) Cl^- distribution and (c) Na^+/K^+ ATPase activity

(*a*) For measurement of alanine transport, hepatocytes were preincubated with 0.2 mM-dibutryryl cyclic AMP for the times shown, and transport was measured over a 2 min time interval. (*b*) In experiments where Cl^- distribution was measured, cells were preincubated for 15 min with $^{36}Cl^-$ plus [^{3}H]inulin, and a sample was taken at zero time. Cyclic AMP (0.2 mM) was then added and radioisotope distribution was measured after the times shown. (*c*) For measurement of Rb^+ uptake, cells were preincubated with 0.2 mM-cyclic AMP for the times shown, $^{86}Rb^+$ was then added and Rb^+ uptake was measured over the succeeding 2 min. The rates were corrected for those in the presence of 1 mM-ouabain. The control Na^+/K^+ ATPase activity was 4.50 nmol/min per mg. The data for the Na^+/K^+ ATPase experiment are taken from Bradford *et al.* (1985). The results shown are representative of three separate experiments in each case.

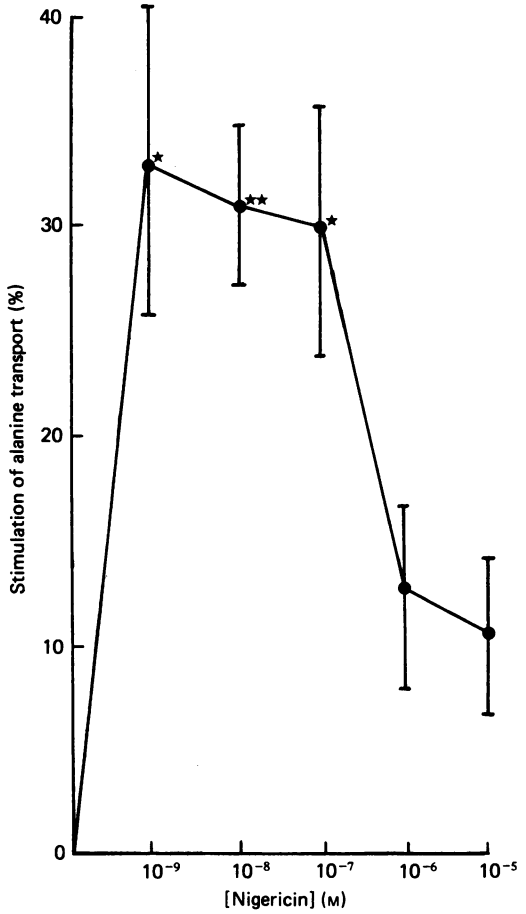

Fig. 5. Dose-dependence of the effect of nigericin on alanine transport

Cells were preincubated with the concentrations of nigericin shown for 10 min before addition of 0.5 mM-alanine, and alanine transport was determined after a further 2 min. The results are shown as means ± S.E.M. of values obtained from four separate cell preparations. The mean control rate of alanine transport in these experiments was 1.61 nmol/min per mg. $*P < 0.5$, $**P < 0.01$ versus control, by a paired t test.

Table 3. Stimulation of alanine transport by nigericin and dibutyryl cyclic AMP and the effect of ouabain

Hepatocytes were preincubated with cyclic AMP (0.2 mM) for 30 min or with nigericin (10 nM) for 10 min before the addition of 0.5 mM-alanine. In some experiments ouabain was added 10 min before the addition of alanine. The initial rate of alanine transport was measured over a 2 min period. The results were obtained from experiments with four separate cell preparations. Significance was assessed by a paired t test: $*P < 0.05$, $**P < 0.01$ versus control; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ versus corresponding incubation in the absence of ouabain.

Additions	Initial rate of alanine transport (nmol/min per mg)
None	1.50 ± 0.06
Cyclic AMP	2.45 ± 0.13**
Nigericin	2.24 ± 0.05**
Cyclic AMP + nigericin	2.53 ± 0.13**
Ouabain	1.17 ± 0.08**†
Cyclic AMP + ouabain	1.19 ± 0.07*††
Nigericin + ouabain	1.19 ± 0.09*††

produced no additional effect. The effects of optimum concentrations of nigericin on alanine transport and membrane potential were thus identical with those of cyclic AMP.

DISCUSSION

This paper demonstrates a correlation between the initial rate of amino acid transport and the cell membrane potential in isolated hepatocytes. The calculation of membrane potential from Cl^- distribution is justified empirically on the following grounds. (i) Cl^- crosses the cell membrane rapidly. (ii) Cl^- permeation is not carrier-mediated, since known inhibitors of Cl^- transport systems are without effect; other investigations have similarly failed to find evidence for carrier-mediated Cl^- transport (Scharschmidt *et al.*, 1982; Bakker-Grunwald,

Table 2. Effects of various hormones on the initial rate of alanine transport, distribution of $^{36}Cl^-$ and Na^+/K^+ ATPase activity in isolated hepatocytes

Hepatocytes (5 mg/ml) were preincubated with the various effectors for 30 min. Cl^- distribution was measured after a further 10 min. Cl^- distribution and the transport of alanine (0.5 mM) were measured in the same cell suspensions. For measurement of Na^+/K^+ ATPase activity, the uptake of $^{86}Rb^+$ was determined over a period of 6 min after the preincubation with the hormones, and the uptake in the presence of 1 mM-ouabain was subtracted. The number of cell preparations for each value is shown in parentheses. Significance was assessed by a paired t test. The control Na^+/K^+ ATPase activity was 4.50 ± 1.5 nmol/min per mg ($n = 4$). $*P \leqslant 0.05$, $**P \leqslant 0.01$, $***P \leqslant 0.001$ versus control.

Additions	Initial rate of alanine transport (nmol/min per mg)	$^{36}Cl^-$ distribution ratio (in/out)	Membrane potential calculated from $^{36}Cl^-$ distribution (mV)	Na^+/K^+ ATPase activity (% of control)
None	1.90 ± 0.36 (4)	0.263 ± 0.011 (4)	−34.8 ± 1.08 (4)	100
Glucagon (300 nM)	2.40 ± 0.28 (4)*	0.211 ± 0.012 (4)*	−40.6 ± 1.50 (4)	131 ± 9 (4) **
Cyclic AMP (0.1 mM)	2.47 ± 0.11 (4)*	0.123 ± 0.012 (3)***	−54.6 ± 2.47 (3)	159 ± 22 (4)*
Vasopressin (25 nM)	1.82 ± 0.43 (3)	0.254 ± 0.008 (3)	−35.7 ± 0.75 (3)	111 ± 6 (3)
Phenylephrine (25 μM)	1.88 ± 0.40 (3)	0.260 ± 0.004 (3)	−35.1 ± 0.34 (3)	107 ± 15 (3)

1983). (iii) The electrogenic permeability of the liver cell membrane is high, since Cl^- is effective in compensating the electrogenic co-transport of Na^+ plus alanine in liver plasma membrane vesicles (Sips *et al.*, 1980*b*). (iv) The potential determined from Cl^- distribution in isolated hepatocytes (Bradford *et al.*, 1985) under several conditions is very close to that previously determined in perfused liver by microelectrode measurements (Claret & Mazet, 1972; Friedmann & Dambach, 1973). At present, there is no satisfactory direct method of measurement of membrane potential in isolated hepatocytes; the values obtained by direct impalement of cultured hepatocytes with microelectrodes do not coincide with those observed in intact liver (Wondergem & Harden, 1980).

The possibility that Cl^- interacts with the alanine carrier and thus stimulates transport directly cannot be ruled out from the present data. However, in liver plasma-membrane vesicles, Na^+-linked alanine transport is not Cl^--dependent. Cl^- stimulates transport only insofar as it acts as a permeant anion in this system; the stimulation by Cl^- is similar to that produced by the permeant anions thiocyanate and nitrate (Sips *et al.*, 1980*a*).

The results presented here are consistent with the postulate that alanine transport is regulated by the cell membrane potential and that the membrane is hyperpolarized by cyclic AMP. It is to be expected that Na^+-dependent transport would depend on the Na^+ electrochemical gradient rather than the membrane potential alone. The intracellular and extracellular Na^+ concentrations were not measured in the present study, and there are considerable technical difficulties involved in measuring intracellular Na^+ concentrations in cells suspended in conventional saline media. Any changes in extracellular Na^+ concentration owing to Na^+ leakage from cells suspended in 150 mM-Na^+-containing medium would be expected to be negligible. Effects of cyclic AMP on intracellular Na^+ concentrations in isolated hepatocytes have not been reported. However, it is probable that the observed changes in membrane potential account for the major part of the change in Na^+ electrochemical potential in these experiments.

No simple relationship between the initial rate of alanine transport and the cell membrane potential is to be expected. According to Geck & Heinz (1976), an increase in the electrochemical potential gradient of Na^+ may affect either the K_m for the co-transported substrate or the $V_{max.}$ of transport, or both parameters. For alanine transport into hepatocytes, the $V_{max.}$ of transport is increased by cyclic AMP, but the K_m is unchanged. On the assumption that the change in Na^+ electrochemical potential caused by cyclic AMP is due mainly to an increase in membrane potential, these results are consistent with the 'affinity' model of Geck & Heinz (1976), where Na^+ changes the affinity of the carrier for alanine and the empty carrier molecule is negatively charged.

It has been argued (Shotwell *et al.*, 1983) that the short-term stimulation of alanine transport is unlikely to be a consequence of increased membrane potential, since transport via the Na^+-dependent systems ASC and N should be similarly affected. It has been reported that transport via these systems is insensitive to hormones (see Shotwell *et al.*, 1983). The experiments in the present paper were performed over short time courses under conditions where the metabolism of the added substrates was negligible. Under these conditions, reagents that affect the cell membrane potential also affect both the transport of glutamine and the *N*-methylaminoisobutyrate-insensitive transport of alanine. These considerations suggest that systems ASC and N are subject to regulation by changes in membrane potential.

It has been shown by Bashford & Pasternak (1984) that the cell membrane potential in Lettre cells is determined by the electrogenic Na^+/K^+ ATPase activity rather than by the K^+ diffusion gradient. In isolated hepatocytes, the change in potential (as calculated from Cl^- distribution) on adding cyclic AMP is abolished by ouabain. The increase in alanine transport activity caused by both cyclic AMP and nigericin is also abolished by ouabain. The liver cell has a rather low permeability to K^+ ions, and the membrane potential measured by microelectrode puncture of perfused liver (-35 mV) is considerably removed from the K^+ diffusion gradient. These observations are consistent with the postulate that Na^+/K^+ ATPase activity is an important determinant of membrane potential in hepatocytes.

The ouabain-sensitive mechanism by which cyclic AMP affects the cell membrane potential remains to be clarified. The effects of cyclic AMP on Cl^- distribution and alanine transport are identical with those of the antibiotic nigericin. The mechanism of action of nigericin is well understood (Pressman *et al.*, 1967; Henderson *et al.*, 1969). Nigericin is a weak acid which cannot cross biological membranes in its anionic form. Nigericin catalyses a net 1:1 electroneutral exchange between any of the cations Na^+, K^+ or H^+. When added to hepatocytes in normal Krebs–Henseleit medium, the overall effect of nigericin is to catalyse a net electroneutral exchange of external Na^+ for internal K^+. The only mechanism by which nigericin can affect electrogenic Na^+-dependent alanine transport is via membrane hyperpolarization brought about by the effect of increased internal Na^+ concentration on the electrogenic Na^+/K^+ ATPase. Therefore, the effects of cyclic AMP could be explained as a stimulation of a so-far undefined electroneutral Na^+/K^+ exchange system in the hepatocyte plasma membrane.

Short-term stimulation of alanine transport is mediated by cyclic AMP and by glucagon, but not by vasopressin and phenylephrine, which do not increase the concentration of cyclic AMP in the liver. It is therefore assumed that the effect of glucagon is mediated via cyclic AMP. This hyperpolarizing effect of cyclic AMP may be specific to the liver. At physiological concentrations of alanine, the rate of transport of alanine exerts significant control on the rate of alanine metabolism (Sips *et al.*, 1980*a*; McGivan *et al.*, 1981; Fafournoux *et al.*, 1983). It has been shown that glutamine transport is similarly important in the regulation of glutamine metabolism when liver glutaminase is activated (Haussinger *et al.*, 1985). The magnitude of the short-term stimulation of alanine transport by glucagon is similar to that of the stimulation of gluconeogenesis from alanine by the hormone (Joseph & McGivan, 1978). It is probable that the short-term stimulation of alanine transport characterized in this paper is of importance in the regulation of gluconeogenesis in the liver.

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