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ALANINE TRANSPORT BY CHINESE HAMSTER OVARY CELLS WITH ALTERED PHOSPHOLIPID ACYL CHAIN COMPOSITION

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Summary

The Na^+-dependent transport of alanine has been examined in Chinese hamster ovary (CHO) cells as a function of the fatty acid composition of their membrane lipids. Significant changes in the fatty acid composition of the CHO cell phospholipids were achieved by supplementation of the growth medium with specific saturated (palmitate) or monoenoic (oleate) free fatty acids. Arrhenius plots of the temperature-dependent uptake of alanine were constructed for cells of altered fatty acid composition. Alanine uptake was characterized by a single discontinuity in the Arrhenius plot. The temperature of this break was observed to be dependent upon the fatty acid composition of the cell phospholipids, ranging from 16°C for cells enriched with oleate to 32°C for cells enriched in palmitate. Calculation of the K_m value for the uptake process showed no significant change with temperature or fatty acid supplementation. Correlations are made between the physical state of the membrane lipids and the temperature-dependence for alanine transport. The results are discussed in terms of membrane fatty acid composition, ordered ⇌ fluid phase transitions and amino acid transport.

Introduction

Many reports exist in the literature which implicate a role for the physical state of the membrane phospholipids in the regulation of a variety of membrane-associated transport or catalytic functions [1—12]. In a number of cases the changes in biological activities have been correlated with the physical state of the membrane lipids. It is our goal in the present work to determine what effect alteration of the fatty acid composition of Chinese hamster ovary (CHO) cell membranes would have on a membrane-associated process. Previous reports

from this laboratory have shown that CHO cells are capable of incorporating significant amounts of exogenous fatty acids into their cellular phospholipids [13,14]. Incorporation of free fatty acids into these membranes was independent of any apparent compensatory changes in polar head-group composition or sterol/phospholipid ratios [13]. Furthermore, we have demonstrated that incorporation of saturated fatty acids into these membrane phospholipids resulted in altered physical properties of the phospholipids as detected by fluorescence intensity and polarization measurements using *cis*- and *trans*-parinaric acid [13]. Fluorescence depolarization studies indicated that the amount of ordered lipid could be increased by appropriate supplementation with palmitic acid. Together, these data strongly suggest that the acyl chain composition of CHO cell membranes could be altered in such a manner as to influence the temperature of the ordered $\rightleftharpoons$ fluid transition in these membrane phospholipids.

A logical extension of this work is to examine what effects changes in membrane fatty acid composition and the physical state of the membrane have on membrane-associated functional processes. A number of precedents exist for this type of study in procaryotic systems. The transport systems for β-glucosides and β-galactosides in unsaturated fatty acid auxotrophs of *Escherichia coli* have been correlated with changes in phospholipid fatty acid composition by several investigators [4—10]. These reports indicated that changes in the transport rate observed as a function of temperature could be related to changes in the physical state of the membrane. In eucaryotic systems the results, to date, have been less well-defined. A number of studies on membrane-associated enzymes have suggested that the composition of the membrane phospholipids (either fatty acid or polar head-group modifications) play a regulatory role in enzyme activity [3,15,16]. However, it has been difficult in many of these systems to correlate changes in activity with an altered physical state of the membrane phospholipids. In other cases, alterations in the phospholipid composition resulted in little or no detectable change in functional properties [16,17].

The Na^+-dependent carrier-mediated transport of alanine has been well characterized in eucaryotic systems by a number of investigators [18—22]. Kaduce et al. [24] have examined the effect of fatty acid supplementation on the transport of α-aminoisobutyric acid, an amino acid analog, in Ehrlich ascites tumor cells. They suggested that changes in transport rates observed as a function of fatty acid supplementation were due to fluidity-induced changes in substrate binding affinities.

This paper will present our observations on the effect of altering the fatty acid composition of membrane phospholipids on alanine transport in CHO cells.

Materials and Methods

Cell culture and growth

Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (ATCC CCL-61). Stock cultures were grown and maintained on Ham's F-12 (Gibco) or minimal Eagle's medium (Flow Laboratories) in a 5% CO_2 atmosphere at 37° C. The medium was supplemented with 5 or 6.6% delipidated serum prepared by the method of Rothblat et al. [25]. As

measured by gas-liquid chromatography, the delipidated serum was shown to contain less than 5% of the serum-associated acyl chains. For uptake experiments, cells were removed from stock flasks and plated at $1 \cdot 10^6$ cells per 100 × 15 mm petri dish containing 12 glass coverslips (9 × 22 mm). The cells were incubated for 48 h (approximately two cell-doublings) on media containing delipidated serum. At this time the cells had reached 50—60% confluency. The media were then changed to one containing the specified concentrations of fatty acids which were added from an ethanolic stock solution. Cells were incubated for an additional 12—17 h. This feeding schedule was designed so that fatty acid incorporation and uptake experiments were completed on sub-confluent cells. Incorporation of fatty acids into the phospholipid fraction reached a maximum in 12 h and remained constant through to 17 h.

Uptake measurements

Uptake of [1-^{14}C]alanine (175 Ci/mol, New England Nuclear) was determined using the procedure of Foster and Pardee [26] as modified by Oxender et al. [19,21] for cells grown on glass coverslips. The uptake buffer contained 0.01 M potassium phosphate, pH 7.4, with 2.5 mM KCl, 0.1% glucose, 0.01% $CaCl_2$ and 0.01% $MgCl_2$. Alanine was present at 0.25 mM (10 μCi/1.1 ml) except as noted. To monitor total uptake of alanine the uptake buffer also included 0.14 M NaCl. The Na^+-indepdent uptake of alanine was measured in the presence of 0.14 M choline chloride (in place of NaCl) which was added to maintain equivalent osmolarity. The Na^+-dependent uptake was taken as the difference between total and Na^+-independent uptake. Prior to the uptake measurements, the cells were incubated for 10—15 min at room temperature in the above buffer to reduce intracellular amino acid levels. Coverslips were then washed three times in either Na^+- or choline-uptake buffer to ensure removal of any non-adherent or non-viable cells. Uptake measurements were then initiated by placing the coverslips in uptake buffer containing labeled amino acid. After the specified time periods, coverslips were removed from the uptake buffer and rapidly washed three times by sequential immersion into beakers of ice-cold buffer. Excess buffer was removed by capillary action when the edge of the coverslip was touched onto a towel. Coverslips were placed in scintillation vials containing 1.0 ml of 0.2 M NaOH. Aliquots of the solubilized cells were removed for protein analysis by the method of Lowry et al. [26]. The cell suspension was neutralized with 0.2 ml of 3.0 M sodium citrate, pH 3, and counted in a Triton-toluene scintillation fluid.

Lipid analysis

A portion of the cells grown on coverslips was washed in phosphate-buffered saline to remove any non-adherent cells. Cells were removed from coverslips by scraping with a rubber policeman, pooled and washed three times with phosphate-buffered saline to ensure removal of growth media. The resultant cell pellet was extracted with chloroform/methanol (2 : 1) and the phospholipid fraction was isolated by silicic acid chromatography. Fatty acid methyl esters were prepared by transesterification in 2% H_2SO_4 in methanol for 60 min at 70°C. Fatty acid methyl esters were identified and quantitated by gas-liquid

chromatography using a model 7610-A Hewlett-Packard gas chromatograph equipped with a 6 ft column of 15% SP2340 on chromosorb P AW-DMCS. Standard fatty acid methyl esters were obtained from Supelco.

Results

Time course and Na^+-dependence of L-alanine uptake by CHO cells

The data shown in Fig. 1 illustrate the time course and Na^+-dependence of *L*-alanine uptake into CHO cells. These data are consistent with the observation that alanine uptake by other eucaryotic cells is predominantly an Na^+-dependent carrier-mediated transport system [18,19]. In the present study we have not distinguished between the 'A' and 'ASC' systems for Na^+-dependent uptake of alanine as described by Christensen [20] and consequently our results could reflect the contributions from both systems. Uptake in the absence of Na^+ in these experiments represents the contribution to total alanine uptake by competing processes such as 'L' system transport, passive diffusion or non-specific absorption. All values presented in this paper have been corrected for Na^+-independent uptake. This uptake was observed to be linear under the indicated conditions for at least 10 min (Fig. 1). In subsequent experiments, 5- or 6-min time points were routinely employed.

Kinetic parameters of alanine uptake

The data presented in Fig. 2 depict the relationship between alanine concentration and the Na^+-dependent and Na^+-independent rates of uptake. In the absence of Na^+, the rate of alanine uptake appeared to increase linearly over the concentration range examined, exhibiting no saturation. The lack of saturation can be accounted for if the major component of Na^+-independent transport is

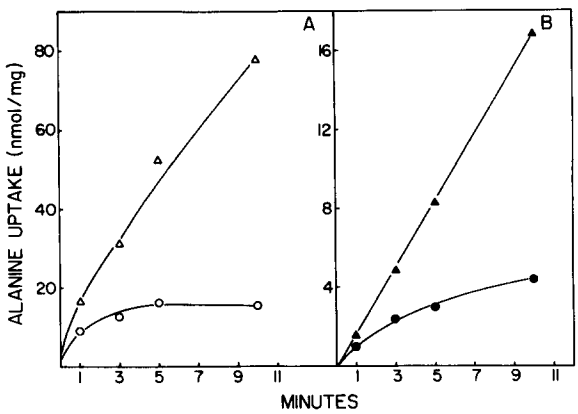

Fig. 1. (A) Time course of alanine uptake by CHO-K1 cells. Uptake experiments were performed with 1.0 mM alanine at 25°C as described in Materials and Methods. At the time of assay, cells were at 70–80% confluency with a mean protein content of 31 μg per coverslip. Cells were assayed in the presence of 140 mM NaCl (△) or 140 mM choline chloride (○). Each point represents the mean of duplicate or triplicate determinations. (B) Time course of alanine uptake with 0.1 mM alanine. Cells were assayed in the presence of 140 mM NaCl (▲) or 140 mM choline chloride (●). Assay conditions as in A.

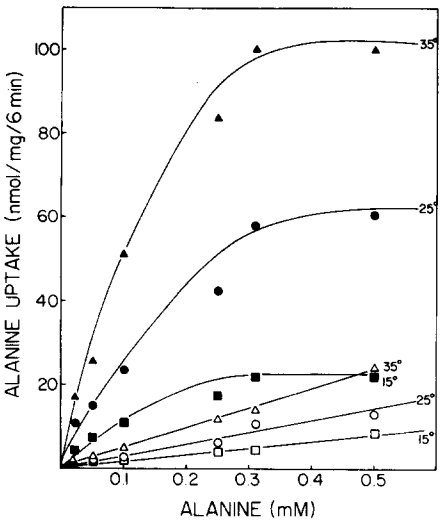

Fig. 2. Concentration-dependence of alanine uptake by CHO cells. The rates of alanine uptake were determined in the presence (▲,●,■) and absence (△,○,□) of 140 mM Na^+ as described in Materials and Methods. The values shown for Na^+ have been corrected for Na^+-independent processes as previously described. Uptake incubations were performed for 6 min at the indicated temperature. Points are the mean of triplicate determinations. Cells were 70% confluent at the time of assay.

passive diffusion or non-specific binding to the cell membrane. In contrast, the Na^+-dependent process showed simple saturation kinetics with saturation occurring at 0.2—0.3 mM. In some experiments, the contribution of Na^+-independent uptake to total uptake was elevated at higher alanine concentrations. To minimize the contribution of Na^+-independent mechanisms, while working close to V, a concentration of 0.25 mM alanine was chosen. The kinetic parameters, K_m and V, were calculated for the Na^+-dependent uptake of alanine as a function of temperature and fatty acid supplementation, and are given in Table I. The apparent K_m for Na^+-dependent uptake was approx. 200 μM. Although there was some variation in the K_m, the difference was in no case greater than 2-fold and was within the error of our measurements. In addition, the variations showed no regular change with either temperature or fatty acid supplement, and it was concluded that the binding affinity of the Na^+-dependent alanine transport system was independent of temperature or fatty acid modification.

Fatty acid supplementation of CHO cell phospholipids

The fatty acid composition of CHO cell membranes was modified by supplementation of the medium with palmitate or oleate, essentially according to the procedure of Rintoul et al. [13]. It had previously been noted that cell growth was inhibited in the presence of 12 μg/ml palmitate, but also that the addition of 2 μg/ml oleate to the palmitate allowed cell division to continue [13]. Our results showed greater incorporation of palmitate into the cellular phospholipids when oleate was present, presumably due to the increased rate of growth under these conditions.

TABLE I

KINETIC PARAMETERS FOR Na^+-DEPENDENT ALANINE TRANSPORT

Kinetic parameters were determined from double-reciprocal plots of alanine uptake. Uptake measurements are described in the legend to Fig. 2 and in Materials and Methods. Cells grown on coverslips to 50—60% confluency were supplemented with palmitate 12 of 17 h prior to the measurement of uptake as described in Materials and Methods. Unsupplemented cells received fresh media with no added fatty acid 17 h prior to uptake measurements. K_m values (mM) at 140 mM Na^+, V values in nmol/mg per min.

Supplement	Temperature (°C)		
	15	25	35
K_m			
none	0.15	0.29	0.22
none	0.16	0.23	0.24
16:0	0.29	0.26	0.25
16:0	0.14	0.12	0.14
V			
none	4.8	16.6	27.7
none	7.2	19.2	25
16:0	4.0	12.5	28
16:0	9.5	18.5	23

The fatty acid compositions of the phospholipids of CHO cells grown under these conditions are given in Table II. CHO cells are characteristic in their relatively simple acyl chain composition, with palmitate and oleate comprising over 70% of the total phospholipid acyl chains. It was observed that upon

TABLE II

ACYL COMPOSITION OF CHO CELL PHOSPHOLIPIDS

Correlations between the temperature of the change in slope of the Arrhenius plot for Na^+-dependent uptake of alanine and the temperature of the phase transition for CHO cell phospholipids as determined by fluorescence measurements using *trans*-parinaric acid. Discontinuities were determined as described in the legend to Fig. 3. Temperatures of the *trans*-parinaric acid (*t*-PnA) fluorescence transitions were extrapolated from the data of Rintoul et al. [13] for extracted CHO cell phospholipids having equivalent ratios of unsaturated to saturated acyl chains. SAT, saturated fatty acid; UFA, unsaturated fatty acid.

Supplement (μg/ml)	Fatty acid composition (%)							SAT	UFA	Transport break point point (°C)	*t*-PnA fluorescence break point (°C)
	14:0	14:1	16:0	16:1	18:0	18:1	18:2				
None	2	—	19	14	12	53	—	33	67	21	20
18:1 (12)	—	—	9	7	15	68	—	24	75	18	15
18:1 (12)	1	—	11	9	20	59	—	32	68	27	19.5
16:0 (10) + 18:1 (2)	—	—	28	7	20	44	—	48	51	29	27.5
16:0 (12)	trace	—	31	20	12	37	—	43	57	25	24.5
16:0 (15) + 18:1 (2)	6	trace	38	14	13	30	trace	57	43	31	32
16:0 (15) + 18:1 (2)	3	—	37	10	17	32	—	57	43	32	32

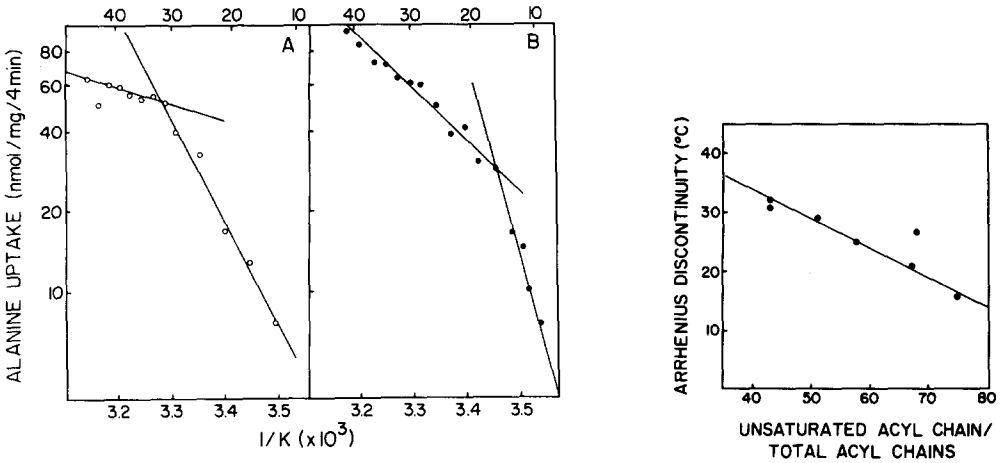

Fig. 3. Arrhenius plots of Na^+-dependent alanine uptake. Alanine uptake was measured as described in Materials and Methods in buffer containing 0.25 mM alanine (10 μCi/1.1 ml). Uptake was measured from 10 to 44°C using 4-min incubations. Values were corrected for Na^+-independent uptake at each temperature as previously described. All points are the mean of triplicate points. The data have been subjected to linear regression analysis. The transition temperature range was selected to give the best fit of lines. Correlation coefficients determined for these lines were greater than —0.95 in all cases. Although drawn as straight lines, the data could also be fitted to a smooth curve. (A) Data obtained from cells supplemented for 15 h in the presence of 15 μg/ml palmitate and 2 μg/ml oleate. (B) Data obtained from cells supplemented with 12 μg/ml oleate for 14 h. All cells were 70% confluent at the time of uptake measurements.

Fig. 4. Unsaturated acyl chains/total acyl chains of CHO cell phospholipids versus the temperature of the change in uptake rate of the Arrhenius plot for Na^+-dependent alanine uptake. The acyl chain composition was calculated by gas-liquid chromatography as described in Materials and Methods. Uptake measurements and determination of the temperature discontinuity of the Arrhenius plot are described in Materials and Methods and Fig. 3.

supplementation with either palmitate or oleate, the supplemented component was enriched at the expense of the other major component. In this manner it was possible to cause significant changes in the ratio of unsaturated to saturated fatty acids present in the CHO cell membranes (see Table II).

Temperature-dependence of Na^+-dependent alanine uptake

The temperature-dependence of the Na^+-dependent alanine uptake was measured on unsupplemented and oleate- or palmitate-supplemented cells. Arrhenius plots were constructed for the uptake process and are shown for one set of palmitate- and oleate-supplemented cells in Fig. 3A and B. In all cases, the Arrhenius plots for alanine uptake were characterized by a single discontinuity in the uptake rate. In the examples given, discontinuities in the Arrhenius plots were observed at approx. 30° C for palmitate-supplemented cells and approx. 20° C for oleate-supplemented cells.

The temperature of the thermal discontinuity was observed to vary with the acyl composition of the CHO cell phospholipids, as summarized in Table II. An even more striking correlation could be made between the ratio of unsaturated acyl chains and the temperature of the Arrhenius break point. This correlation is illustrated in Fig. 4. As the percent of unsaturated acyl chains increases in the CHO cell phospholipids, a decrease in the temperature of the Arrhenius discon-

tinuity is obtained. The anomalous behavior noted in Fig. 4 at 68% unsaturation is inexplicable.

A similar correlation has been made between the ratio of unsaturated acyl chains and the temperature of the Arrhenius break points as determined by *trans*-parinaric acid fluorescence measurements of CHO cell phospholipids [13]. Using this relationship and the fatty acid compositions shown, the temperature of the fluorescence break point was extrapolated as given in Table II. The Arrhenius break temperatures determined by these independent methods were found to be almost equal.

Discussion

The results demonstrate that the rate of Na^+-dependent alanine transport in CHO cells can be altered upon modification of the fatty acid composition of CHO cell phospholipids. Supplementation of the growth media of CHO cells with either oleate or palmitate resulted in the incorporation of these fatty acids into the cell phospholipids. The incorporation was not accompanied by any significant alterations in the polar head-group composition or the phospholipid to sterol ratio [13]. The consequence of this incorporation was a variation in the ratio of unsaturated to saturated acyl chains of 43—75% in these studies. A clear correlation could be made between this ratio and the discontinuity in the Arrhenius plot for Na^+-dependent alanine transport. Furthermore, the temperature of the discontinuity of the Arrhenius plot could be correlated with the thermal phase transition of CHO cell phospholipids as monitored by *cis*- or *trans*-parinaric acid fluorescence [13]. This correlation is particularly good in the case of control and palmitate-supplemented cells, with variation within 2°C between the temperatures of the Arrhenius discontinuity for transport and the thermal phase transition of the cell phospholipids. The correlation is less exact in the case of oleate-supplemented cells. The reason for this variation is not known. We feel these correlations provide evidence for a role of the physical state of the membrane in the function of this amino acid transport system.

Mechanistically there are numerous interpretations for a change in reaction rate described by the Arrhenius equation. These interpretations fall into two general categories for cases involving lipid modification. The first evokes fluid to ordered phase transitions of the phospholipids in the membrane. Changes in reaction rate could be accounted for by a partitioning of the protein into regions of altered mobility or restriction. An alternative explanation involves changes in the intrinsic properties of the protein (i.e., binding affinities, specific activation by fatty acid moiety). To test the latter possibility, the kinetic parameters of the uptake process were examined as a function of temperature and fatty acid supplementation. The K_m value for uptake did not vary significantly. In cases where changes in the binding affinity have been related to discontinuities in the Arrhenius plot, the temperature-induced changes in K_m have been over 10-fold [28,29].

In only a few cases have eucaryotic transport systems been examined as a function of in vivo fatty acid modifications. Kanduce et al. [24] have examined α-aminoisobutyric acid uptake in Ehrlich ascites cells with altered fatty acid composition. They observed single discontinuities in the Arrhenius plot at

approx. 30°C regardless of supplement. Although they achieved significant alteration in the ratio of monenoic to polyenoic fatty acids upon dietary supplementation, there was no appreciable change in the ratio of saturated to unsaturated fatty acids in these membranes. Based on our results for the correlation between unsaturated/total acyl chain composition to changes in the Arrhenius discontinuity, this result is not unexpected. Correlation with physical measurements has not been made on these membranes. In comparison, Wisnieski et al. [30] observed six breaks in the Arrhenius plot for α-aminoisobutyric acid transport in LM cells. These breaks were correlated with five characteristic temperatures detected by ESR. The physical interpretation of these results remains unclear.

In summary, the results presented here provide evidence for an interrelationship among the acyl chain composition of the membrane, amino acid transport and physical state of the membrane phospholipids as reported previously [13]. The agreement in the temperature-dependent changes in transport and physical properties suggests an intimate association of the carrier protein with the phospholipid of the bilayer. Since the fluorescence measurements were determined on phospholipid preparations in the absence of neutral lipids, it might be suggested that the carrier protein is localized in a cholesterol-deficient region of the membrane.

Verification of this idea and the precise protein-lipid interactions involved must await the resolution of this transport system and its reconstitution into a more-defined lipid environment. Our results suggest that the Na^+-dependent transport system for alanine uptake would be a reasonable candidate for further investigation.

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