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Enhanced Transport of Natural Amino Acids after Activation of Pig Lymphocytes

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Na^+-dependent uptake of the amino acids L-proline and L-methionine was greatly accelerated when pig lymphocytes were activated with phytohaemagglutinin or other mitogens. The increased influx was apparent after incubation with phytohaemagglutinin for 1h, and reached a maximum after 24h. The lymphocytes appear to possess at least three different transport systems for neutral amino acids with properties similar to, but not identical with, those described for other cells. The activity of a system resembling the A system of other cells was increased most dramatically after activation, its activity in unstimulated lymphocytes being extremely low or absent. A second Na^+-dependent system, which has properties similar to those of the ASC system in other cells, but with a broader specificity for amino acids, was more active in unstimulated lymphocytes, and uptake by this system was also accelerated after incubation with phytohaemagglutinin. The activity of a third system, very similar to the L system in other cells, was increased to a much smaller extent after lymphocyte activation.

The addition of mitogenic lectins to cultured lymphocytes results in a rapid increase in the rate of transport of many different metabolites across the cell membrane (Ling & Kay, 1975). Amongst the changes observed is an increase in the rate of uptake of the non-metabolizable amino acid analogue 2-aminoisobutyric acid (Mendelsohn *et al.*, 1971; Van den Berg & Betel, 1973*a*). Similar increases in amino acid transport are seen when many different types of mammalian cells respond to proliferative or hormonal stimuli (Guidotti *et al.*, 1978).

Most types of mammalian cell have at least three different systems responsible for the transport of neutral amino acids, the L, A and ASC systems (Christensen, 1975; Guidotti *et al.*, 1978). The L system can be distinguished from the others by virtue of its lack of dependence on extracellular Na^+, whereas the ASC system can be differentiated from the A system by its inability to transport *N*-methylated amino acids (Christensen *et al.*, 1967; Christensen, 1969). The three transport systems usually have rather different amino acid specificities, but both these specificities and the relative activities of the three systems may vary from one type of cell to another.

Previous studies on the responses of amino acid transport in lymphocytes to the addition of mitogens have concentrated on the uptake of non-metabolizable amino acids, such as 2-aminoisobutyric acid and 1-aminocyclopentanecarboxylic acid, usually with the tacit assumption that they are transported by specific systems originally described in other cells. Stimulation of Na^+-dependent uptake of 2-aminoisobutyric acid after addition of mitogens has been clearly demonstrated in those studies, but the response of Na^+-independent systems was somewhat equivocal because no measurements were made in the absence of Na^+ (Van den Berg & Betel, 1973*a*,*b*). To provide a more comprehensive picture we have examined the changes in uptake of some natural amino acids after activation of lymphocytes by mitogens and have found that, although the cells appear to possess at least three different transport systems for amino acids, their properties are not identical with those described for other mammalian cells. Amino acid uptake by all three systems was accelerated after mitogen addition, but not to the same extent.

Experimental

Materials

L-[5-^{3}H]Proline (sp. radioactivity 24Ci/mmol) and L-[^{35}S]methionine (sp. radioactivity 105Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and unlabelled amino acids from Sigma Chemical Co., St. Louis, MO, U.S.A., except for 2-(methylamino)isobutyric acid, which was a gift from Professor Raffaello Fusco (Department of Chemistry, University of Milan, Milan, Italy). Phytohaemagglutinin-P was purchased from Difco Laboratories, Detroit, MI, U.S.A.,

concanavalin A from Miles Laboratories, Elkhart, IN, U.S.A., and leucoagglutinin, soya-bean lectin and wheat-germ agglutinin from Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation and incubation of lymphocytes

Lymphocytes were purified from defibrinated pig blood as described by Kay *et al.* (1975). The cells (2×10^6/ml) were incubated in Eagle's Minimal Essential medium supplemented with 15% autologous serum for 20–24h with or without addition of 10μg of phytohaemagglutinin-P/ml.

Determination of amino acid uptake

Lymphocytes incubated with or without mitogen were centrifuged at 400*g* for 10min and resuspended in Earle's Balanced Salt solution supplemented with 0.1% glucose for 15min at 37°C. The cells were then again collected by centrifugation and resuspended at 10^8 cells/ml in incubation medium (150mM-NaCl; 5mM-KCl; 1.8mM-$CaCl_2$; 1mM-$MgSO_4$; 1mM-KH_2PO_4 and 20mM-Tris/HCl, pH7.5, at 37°C). In experiments where the Na^+-dependent amino acid uptake was to be determined, the NaCl in the incubation medium was replaced by choline chloride for one set of determinations. Initial rates of amino acid uptake were then determined by warming 0.1ml of cell suspension for 1min, adding 10μl of a solution containing 0.5μCi of radioactive amino acid diluted with unlabelled amino acid to give a final concentration of 0.05 or 0.1mM, and incubating at 37°C for 3–10min.

The uptake was stopped by chilling the tubes in ice and adding 2ml of ice-cold incubation medium. The cells were washed twice in 1.5ml of incubation medium, and then 0.5ml of cold 10% trichloroacetic acid was added to the cell pellet. The mixture was shaken vigorously, the trichloroacetic acid-insoluble material was removed by centrifugation and the radioactivity in 0.25ml of the trichloroacetic acid-soluble extract was determined in a Beckman LS233 scintillation counter. More than 90% of the radioactivity taken up was present in this fraction. The counting efficiency, determined by internal standardization in each experiment, was about 20% for ^3H and 70% for ^{35}S. All values were corrected for the amount of extracellular radioactivity trapped in the cell pellet, determined in each experiment by addition of radioactive isotope to cells maintained at 0°C and then processed as described above. Four or five replicate determinations were made for each value, and results are expressed as pmol of amino acid taken up/10^7 cells per min, ± the standard error of the mean.

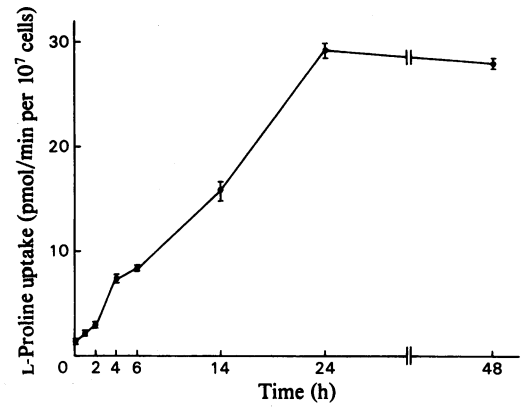

Fig. 1. *Initial rates of uptake of* L-*proline by lymphocytes preincubated with phytohaemagglutinin-P for different periods*

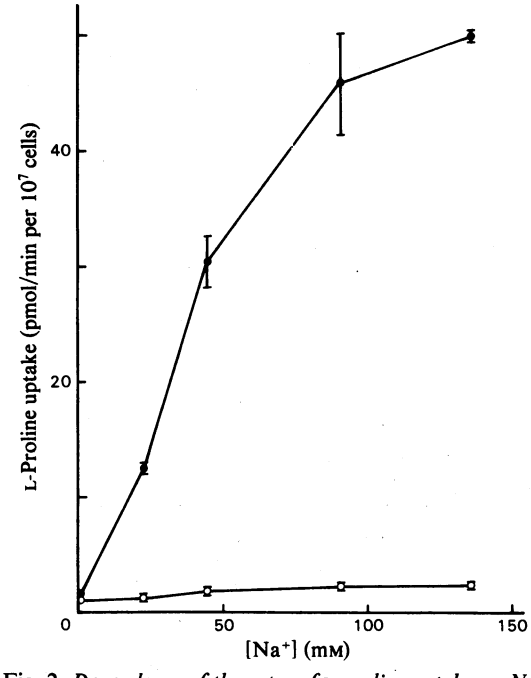

Fig. 2. *Dependence of the rates of* L-*proline uptake on Na^+ concentration*

The initial rates of uptake of 0.05mM-L-proline were determined for unstimulated lymphocytes (○) or cells preincubated with phytohaemagglutinin-P for 18h (●).

Results and Discussion

General effects of mitogens on the uptake of L-*proline*

Incubation of lymphocytes with phytohaemagglutinin-P resulted in a progressive increase in the rate of uptake of L-proline. The influx increased as soon as 1h after addition of mitogen and reached a maximum after 24–48h (Fig. 1). The greatest stimulation of proline uptake was seen when the optimum mitogenic concentration of phytohaemagglutinin-P

was used, and studies with a range of mitogenic and non-mitogenic lectins showed a close correlation between mitogenicity and enhanced proline uptake (results not shown). Stimulation of the influx of this natural amino acid may therefore be regarded as a characteristic feature of lymphocyte activation by mitogenic lectins.

Na^+-dependency and kinetics of the uptake of L-*proline*

With the concentrations of proline normally used (0.05–0.1 mM) only about 60% of its uptake by unstimulated lymphocytes was Na^+-dependent, whereas more than 90% of its greatly increased influx into phytohaemagglutinin-P-activated cells dependedon the presence of extracellular Na^+ (Fig. 2). A kinetic analysis of proline uptake by unstimulated lymphocytes shows that the Na^+-dependent component represents uptake by a saturable process (Fig. 3*a*), although at high concentrations of the amino acid most of the uptake occurred via a non-saturable Na^+-independent route, as shown by the curvilinear line for total uptake in Fig. 3(*a*).

Analysis of the Na^+-dependent component of proline influx into phytohaemagglutinin-P-activated cells reveals that the stimulation of amino acid influx resulted from a marked increase in $V_{max.}$, with no significant change in K_m (Fig. 3*b*). Thus in the experiment shown the K_m was 1.4 mM for unstimulated lymphocytes (Fig. 3*a*), 1.8 mM after incubation with phytohaemmagglutinin-*P* for 4h, and 1.8 mM after 24h (Fig. 3*b*). In all determinations made the observed K_m values were between 1.2 mM and 2.0 mM. In contrast, the observed $V_{max.}$ (in pmol/min per 10^7 cells) for L-proline uptake by unstimulated lymphocytes in different experiments varied from 25 to 81, compared with 155–199 pmol/min per 10^7 cells after incubation with phytohaemagglutinin-P for 4h and 862–1447 pmol/min per 10^7 cells after 24h. Hence the increase in $V_{max.}$ produced by phytohaemagglutinin-P activation was between 10- and 50-fold, the variation being largely due to the variability of $V_{max.}$ in unstimulated lymphocytes.

The results of the kinetic analyses of the Na^+-dependent uptake of L-proline therefore agree with those reported for the effects of mitogenic activation of lymphocytes on their uptake of 2-aminoisobutyric acid (Mendelsohn *et al.*, 1971; Van den Berg & Betel, 1973*b*; Greene *et al.*, 1976), except that the increase in $V_{max.}$ in the present studies was much greater, due to more careful correction for the Na^+-independent component.

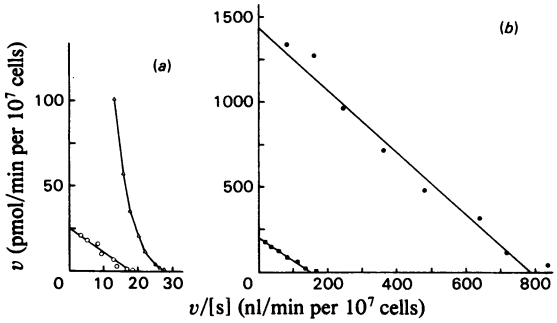

Fig. 3. *Kinetic analysis of the Na^+-dependent component of* L-*proline uptake*

The initial rates of uptake of L-proline were determined in the presence and absence of Na^+, and data analysed by the Eadie–Hofstee method. Total (△) and Na^+-dependent (○) uptake by unstimulated lymphocytes are shown in (*a*) and Na^+-dependent uptake by lymphocytes preincubated with phytohaemagglutininin-P for 4h (■) or 24h (●), on a different scale, are shown in (*b*).

Inhibition of L-*proline uptake by other amino acids*

Studies of the mutual inhibitory effects of different amino acids on their uptake by cells can reveal heterogeneity in transport systems not detectable by other kinetic studies (Christensen, 1966) and such inhibition analyses have revealed the existence of a number of apparently distinct transport systems for amino acids in a variety of cells (Christensen, 1975). We therefore examined the effects of addition of high concentrations of several other amino acids on the influx of L-proline into phytohaemagglutinin-P-activated lymphocytes (Table 1). Only total uptake was measured because more than 90% of this was Na^+-dependent under the conditions used (Fig. 2).

All the amino acids tested except those with positively charged side chains inhibited proline uptake substantially. The strongest inhibition was caused by serine, methionine, alanine and glycine, as expected for transport by an Na^+-dependent system, but substantial inhibition was also produced by amino acids with hydrophobic side chains, such as isoleucine and phenylalanine, which use primarily the Na^+-independent L system in other cells (Christensen, 1975). Hence the Na^+-dependent transport system for proline in lymphocytes has an unusually broad specificity. Equally surprising was the finding that 2-(methylamino)isobutyric acid, a non-metabolizable amino acid analogue that is transported only by the Na^+-dependent A system in most other cells and is the best competitive inhibitor of proline transport in several types of mammalian cells (Christensen, 1975), caused only modest inhibition of proline uptake by the activated lymphocytes (Table 1). This observation therefore shows that the Na^+-dependent uptake of proline by phytohaemagglutinin-P-activated lymphocytes must have been mediated by two distinct systems, one inhibitable by 2-(methylamino)isobutyric acid (A system) and another insensitive to inhibition by 2-(methylamino)isobutyric acid (ASC system).

Table 1. *Inhibition of* L-*proline uptake by phytohaemagglutinin-P-activated lymphocytes by other amino acids*
The effects of individual amino acids, each added at 10 mM, on the initial rate of uptake of 0.1 mM-L-proline are shown in Expt. 1. The results of a second experiment in which the inhibitor/substrate ratio was increased to 200 by a decrease in the L-proline concentration to 0.05 mM are also summarized. Mean values (±S.E.M.) for four determinations are given.

	Expt. 1		Expt. 2
Inhibitor	Proline uptake (pmol/min per 10^7 cells)	Inhibition (%)	Inhibition (%)
None	23.0±1.0	—	—
Ser	4.3±0.1	81	—
Met	—	—	88
Ala	5.2±0.4	77	88
Gly	6.9±0.1	70	75
Leu	7.6±0.4	67	64
Hyp	8.0±0.2	65	—
Trp	8.4±0.3	63	—
Thr	9.3±0.7	60	—
His	9.4±0.4	59	—
2-(Methylamino)isobutyric acid	9.6±1.0	58	65
Ile	11.2±0.3	52	—
Phe	11.3±0.5	51	50
Val	11.4±0.3	51	—
Arg	18.1±0.6	21	—
Lys	—	—	10

The effect of 2-(methylamino)isobutyric acid on the Na^+-dependent component of uptake of proline by both unstimulated and phytohaemagglutinin-P-activated lymphocytes was then examined. No significant inhibition was detected in the unstimulated cells, the mean influxes (in pmol/min per 10^7 cells) being 2.6±0.3 (S.E.M.) without 2-(methylamino)-isobutyric acid and 2.3±0.3 (S.E.M.) in the presence of 2-(methylamino)isobutyric acid, for four replicate measurements. The corresponding values for the phytohaemagglutinin-P-activated cells were 35±1 and 11±3 (mean±S.E.M.) pmol/min per 10^7 cells. Hence it seems that although the ASC system was functioning in the unstimulated cells, and was activated about 4.7-fold by incubation with phytohaemagglutinin-P, the A system was detectable only in the phytohaemagglutinin-P-activated cells.

Differential activation of different transport systems by phytohaemagglutinin-P

Since the last conclusion was based on the study of the very small Na^+-dependent influx of proline into unstimulated lymphocytes, and since the Na^+-independent L system could not be examined satisfactorily with the use of proline, we also investigated the uptake of another natural amino acid, L-methionine, which in other cells is a good substrate for both the A and L systems but not the ASC system (Christensen, 1975). The effects of five natural amino acids and 2-(methylamino)isobutyric acid on both the Na^+-dependent and the Na^+-independent components of uptake of methionine by unstimulated and phytohaemagglutinin-P-activated lymphocytes are shown in Table 2.

The Na^+-independent component of methionine influx exhibited properties characteristic of uptake via the L system in both unstimulated and phytohaemagglutinin-P-activated cells, being greatly inhibited by phenylalanine, valine and leucine, but completely unaffected by proline or 2-(methylamino)-isobutyric acid. Alanine caused partial inhibition. The effect of activation by phytohaemagglutinin-P was to stimulate influx via this system by about 50%. Van den Berg & Betel (1973*a*) reported a much larger increase (about 200%) in the influx of 1-amino-cyclopentanecarboxylic acid into rat lymphocytes after exposure to concanavalin A for 24h, but it is possible that part of that uptake was not mediated by the L system, because no measurements of influx in completely Na^+-free media were made.

The response of the Na^+-dependent component of methionine uptake to activation of the cells by phytohaemagglutinin-P was much more dramatic, showing a 7-fold stimulation (Table 2). Furthermore, the effects of 2-(methylamino)isobutyric acid paralleled those seen with proline uptake, there being no significant inhibition of the methionine influx into the unstimulated cells by 2-(methylamino)isobutyric acid, but about 50% inhibition with the phytohaemagglutinin-P-activated cells. Hence the ASC system again seemed to account for all the Na^+-dependent uptake of the amino acid in the unstimulated cells, and its activity increased about 4.4-fold after mitogen addition. Also in keeping with the results for proline

Table 2. *Comparison of inhibitory effects of amino acids on the uptake of* L-*methionine by unstimulated and phytohaemagglutinin-P-activated lymphocytes*

Pig lymphocytes were incubated for 24h in the presence or absence of phytohaemagglutinin-P before the Na^+-dependent and Na^+-independent components of L-methionine uptake were determined as described in the text. The concentrations of L-methionine and inhibitory amino acids were 0.1 and 10mM respectively. Mean values (±S.E.M.) for four measurements are given.

	L-Methionine uptake (pmol/min per 10^7 cells)							
	Unstimulated				Phytohaemagglutinin-P (24h)			
Inhibitor	Independent of Na^+	Inhibition (%)	Dependent on Na^+	Inhibition (%)	Independent of Na^+	Inhibition (%)	Dependent on Na^+	Inhibition (%)
None	52.4±2.5	—	14.2±2.9	—	76.5±1.8	—	104.1±3.5	—
2-(Methylamino)isobutyric acid	53.1±3.8	0	12.0±1.4	15	81.9±0.6	0	52.7±1.1	49
Pro	53.1±0.4	0	1.7±0.7	88	77.7±1.5	0	19.2±3.2	82
Ala	33.1±2.6	37	0.8±0.9	95	29.0±4.0	62	0	100
Val	9.0±0.5	83	3.5±0.9	75	8.1±1.1	89	7.6±2.1	93
Leu	6.4±0.1	88	1.6±0.2	89	6.1±0.5	92	5.6±1.0	95
Phe	5.6±0.3	89	5.8±0.6	59	5.6±0.4	93	36.7±3.0	65

was the finding that all the natural amino acids tested produced substantial inhibition of Na^+-dependent methionine influx by both systems A and ASC (Table 2), which again illustrates the unusually broad specificity of these systems in lymphocytes (cf. Christensen *et al.*, 1967; Christensen, 1975).

Our results therefore show that the A system is either absent or inactive in the unstimulated lymphocyte, but is quickly formed or activated after addition of mitogen. This suggests that the uptake of 2-aminoisobutyric acid by unstimulated lymphocytes, reported previously by other workers, is probably mediated by the wide-specificity ASC system, rather than by the A system, as has been assumed previously (Mendelsohn *et al.*, 1971; Van den Berg & Betel, 1971, 1973*a*,*b*; Greene *et al.*, 1976). The distribution of Na^+-dependent influx of amino acids between the A and ASC systems in phytohaemagglutinin-P-activated lymphocytes also bears on the kinetic analyses. For example, the observed K_m and $V_{max.}$ values for Na^+-dependent proline influx must represent compound values for both ASC and A systems. But the straight lines obtained (Fig. 3*b*) also show that the individual K_m values for each system could not have been widely different.

It is not easy to reconcile our findings with those of Wettenhall & London (1974), who could not detect significant changes in the uptake of glycine or phenylalanine by pig lymphocytes after they had been activated with concanavalin A. At present the most likely explanation of this apparent discrepancy is that initial rates of uptake were not measured in the latter experiments, so that small changes could easily have been missed. From our results little change in the influx of phenylalanine would have been expected under the conditions used, but some enhancement of glycine uptake would have been predicted.

The parallelism between the increase in activity of Na^+-dependent transport systems for amino acids during lymphocyte activation and the decrease in their activities during maturation of mammalian reticulocytes to erythrocytes (Winter & Christensen, 1965; Wheeler & Christensen, 1967; Antonioli & Christensen, 1969) is marked, but the particular importance of these transport systems in metabolically active cells is unclear. The increasing evidence in favour of Na^+-dependent amino acid transport being an electrogenic process (Eddy, 1977) suggests that changes in the membrane electrical potential could well be an important factor in the regulation of such processes, and it is possible that interaction of mitogens with the cell membrane induces changes in membrane potential.

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