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Amino acid stimulation of alkali-metal-independent ATP cleavage by an Ehrlich cell membrane preparation

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SUMMARY

A stimulation by certain amino acids of the hydrolysis of ATP by a vesicular preparation from the Ehrlich ascites tumor cell has been observed, independently of the presence or absence of Na^+, K^+ or ouabain. This membrane preparation failed to show evidence for the presence of a characteristic amino-acid-stimulated, Na^+–K^+-dependent ATPase previously observed for other preparations. Stimulating activity is shown by two groups of abnormal substrates of Na^+-independent transport System L, which elicit a structure-dependent energization of that system. For some of these amino acids, adding an ionophore served to bring out the stimulation of ATP cleavage.

We have been surprised to observe that a second amino group can be introduced into various neutral amino acids without eliminating their transport by the neutral amino acid systems of the Ehrlich cell, provided that the group has a rather low pK_a, so far not above 8.4. On the contrary, the modified amino acids show accelerated uptake, and strong asymmetry between uptake and exodus[1–4]. This effect is especially impressive for transport System L because the uptake of the normal substrates by this system is not strikingly concentrative, whereas it concentrates the diamino acids under discussion from 10 to 40 times. Furthermore, the accumulation of these amino acids is particularly sensitive to conditions that strongly decrease the ATP level, *e.g.* the presence of 2,4-dinitrophenol or oligomycin during aerobic metabolism[1–4]. We have observed that the presence of two sidechains, as in α,α-diethylglycine, also causes sharp flux asymmetry with high accumulation in the Ehrlich cell. Furthermore, this amino acid because of its especially strong response to H^+ has guided us to evidence for co-transport of H^+ with amino acids by System L

Abbreviations: EGTA, ethyleneglycol-bis(aminoethyl)-tetraacetic acid; MPA, 4-amino-1-methylpiperidine-4-carboxylic acid.

In attempting to explain the above behavior[1-4], we have now observed that amino acids of both these classes, along with more ordinary basic amino acids, stimulate an ATP-splitting activity in a vesicular preparation derived from the Ehrlich cell. This action is obtained whether Na^+ and K^+, or ouabain, are present or not, although the ATPase activity does depend on the presence of Mg^{2+}. The stimulation of the vesicular ATPase by azaleucine, and by diethylglycine, however, could be shown only in the presence of such ionophores as valinomycin, nigericin or gramicidin D.

Although amino-acid stimulation of preparations of the Na^+ and K^+-dependent ATPase of membranes has been reported on at least four occasions[5-8], beginning with Skou, we are not aware that anyone has previously described an ATP-splitting activity specifically stimulated by an amino acid, independent of the presence of Na^+, K^+, or ouabain. Indeed in each previous case, stimulating activity even stronger than that of the amino acids could be observed with EDTA or ethyleneglycol-bis(aminoethyl)-tetraacetic acid (EGTA), substances better known of course for their chelating activity than for their status as somewhat remote analogs of glycine. Accordingly, the prior reports usually attributed the action of the amino acids (in each case including histidine) to an incidental binding of an otherwise inhibitory metal ion, and only the recent report of Forte *et al.*[8] tends to associate the amino acid effect with the biological energization of amino acid transport. In that case a relation to the Na^+-dependent transport activity is proposed.

Method of preparation of membrane fraction: Ehrlich ascites tumor cell were washed 1 to 3 times with Krebs–Ringer bicarbonate medium. An additional wash used 0.25 M sucrose containing a 0.016 M Tris–glycylglycine buffer, pH 8.0, Ca^{2+}= 2 mM. The cells were suspended in three volumes of a similar medium containing 0.012% deoxycholic acid, and the suspension quickly frozen in a solid CO_2–ethanol mixture. On thawing in an ice bath, the suspension was treated with 8 to 10 strokes of a tight-fitting Dounce homogenizer. The temperature was not permitted to rise appreciably above 0 °C during these procedures.

Nuclei were sedimented by centrifuging for 10 min at 2000 rev./min in a refrigerated International Centrifuge. Homogenization of the sediment was repeated in the sucrose–Tris–glycylglycine solution, this time not including deoxycholic acid. The two supernatants were combined and centrifuged 10 min at 12 000 × *g*. Ethyleneglycol-bis-(aminoethyl)-tetraacetic acid (EGTA) was then mixed with the supernatant suspension to a final concentration of 4 mM, and the solution allowed to incubate 1 h at 4 °C, before centrifuging 20 min at 2500 rev./min in the refrigerated International Centrifuge. Finally the supernatant solution obtained was centrifuged 90 min at 48 000 × *g* and the pellet resuspended in a small volume of Tris or Krebs–Ringer bicarbonate buffer, and kept at most for 2 days at 4 °C before study.

A negative-contrast electron photomicrograph kindly made by Dr Robert Gray showed a mainly vesicular character. The stimulation of the cleavage of ATP is illustrated in Table I, with and without the alkali-metal ions and with and without ouabain. The absence of stimulation by oligomycin is also illustrated. The transport of the test amino acid, here 4-amino-1-methylpiperidine-4-carboxylic acid (MPA), has been characterized

TABLE I

RELEASE OF ^{32}P FROM ATP BY THE MEMBRANE PREPARATION

Included 3 ml: (γ-$^{32}P_i$)ATP–Tris salt, 4 mM; an amino acid, as listed, at 8 mM; $MgCl_2$, 7 mM; membrane protein, 0.8 to 1.2 mg/ml; Tris buffer, 30 mM, pH 7.5; and 2-mercaptoethanol, 10 mM (ref.9). Incubation 20 min at 37°C, terminated by adding a mixture of 3 ml 10% trichloroacetic acid + 1 ml ethanol. Liberated ^{32}P was measured according to Blostein[10]. Separate tests showed that orthophosphate was released during the incubation.

Addents	*μmoles ^{32}P released/mg protein per h*
None	0.66
MPA	2.07
MPA + $5 \cdot 10^{-4}$ M ouabain	2.20
MPA + NaCl, 100 mM, KCl, 20 mM	2.56
MPA + NaCl, KCl and ouabain	2.13
MPA + oligomycin, 10 μg/ml	1.95

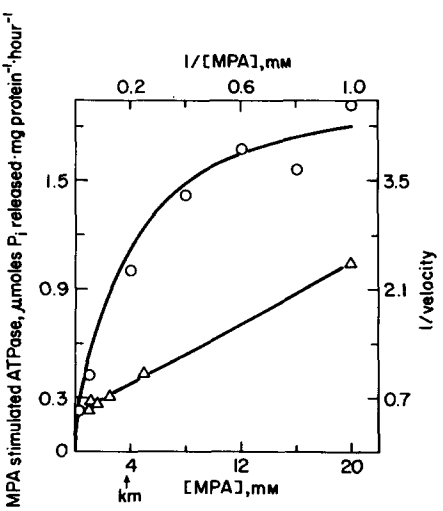

Fig.1. Estimation of apparent K_m for MPA in stimulating membrane-bound ATPase activity in the presence of Mg^{2+} and the absence of Na^+ and K^+. The upper curve relates to the scales at the bottom and left, the straight line is a Lineweaver–Burk plot relating to the scales at the top and the right.

as strongly concentrative and largely due to System L (ref.1). Fig.1 shows that the effect of MPA is half-maximal at about 4 mM. Ordinary substrates of System L (the norbornane amino acid, phenylalanine, methionine, tyrosine, norleucine) showed no significant stimulation of ATP cleavage, nor did EGTA.

Table I shows only small stimulation of ATP cleavage on the addition of Na^+ and K^+, with a correspondingly small inhibition by ouabain. A more sensitive assay for an amino acid stimulation of the kind reported by Forte *et al.*[8] could be made by testing for the phosphatase action of the preparation on *p*-nitrophenylphosphate. Of 19 amino acids tested,

only two showed small stimulating effects under the conditions of this assay*; glutamate by 12% and leucine by 22%. Results for glycine, alanine, proline, threonine, methionine, phenylalanine, histidine, lysine, and homoarginine, also for α-aminoisobutyric acid and several model amino acids, were in the range of 79 to 108% of the background activity. No stimulation was obtained with EGTA at 0.4 mM. These results indicate that our preparation, perhaps fortuitously, does not contain appreciable amounts, at least not in an accessible state, of the ATPase activity studied by Forte *et al.* In addition to the amino acids shown, the isomeric forms of 1,4-diaminocyclohexanecarboxylic acid were effective stimulators.

Concern about the full accessibility of the ATPase activity in the vesicles led us to test the effects of adding various ionophores (Table II). Of the amino acids studied, the migration of α,α-diethylglycine has the largest influence on the H^+ distribution and is the most sensitive to the pH gradient across the Ehrlich cell membrane. Conceivably, there-

TABLE II

STIMULATION OF ATP CLEAVAGE BY THE MEMBRANE PREPARATION IN THE PRESENCE OF IONOPHORES

The amino acids in the first experiment were at 8 mM in 25 mM Tris buffer, and in the second experiment, at 4 mM in a 25 mM choline bicarbonate buffer, pH 7.5. Other details as in Table I.

Amino acid	*Tris buffer*			*HCO_3^- buffer ouabain 0.5 mM nigericin 10 μM*
	Valinomycin 4 μg/ml	*Gramicidin D 10 μM*	*Nigericin 10 μM*	
None	1.48	1.77	1.44	1.53
Ornithine	1.78	1.88	1.98	2.33
Homoarginine	–	–	–	1.97
MPA	2.94	2.17	1.78	2.76
Azaleucine	1.25	2.40	1.65	2.72
Diethylglycine	3.68	2.71	3.19	2.90

fore the ionophores may permit continued amino acid pumping by relieving the effects of the accumulation of co-transported H^+. Such diamino acids as MPA, thialysine, and *cis*-1,4-diaminocyclohexanecarboxylic acid are suspected of serving proton donors to enhance their own transport[4]. Other possible explanations for the present effects of the ionophores will need to be considered.

We may note that such ordinary basic amino acids as ornithine and arginine and their immediately higher homologs also stimulate moderately the cleavage of ATP, although none of these amino acids is greatly concentrated by the Ehrlich cell[10]. The arginine analog, 4-amino-1-guanylpiperidine-4-carboxylic acid[11] has a stronger effect. Although

* The reaction proceeded in 0.05 M Tris buffer, pH 7.5, at K^+, 20 mM; $MgCl_2$, 7 mM; *p*-nitrophenylphosphate, 4 mM, and was terminated by adding 3 ml 10% trichloroacetic acid. The solution was then alkalinized by adding 2 ml 1.2 M Tris, and the absorbance read at 400 nm.

the observed activity otherwise might serve well in explaining a structure-dependent energization of Na^+-independent System L as obtained with these so-called "gradient-sensing" substrates[4], we are not yet prepared to account fully for the distribution of stimulating activity among amino acids. The observed activity appears so far to be independent of any previously known ATPase. It may be noted that many of the observations were made in a 25 mM bicarbonate buffer, with results similar to those observed in Tris buffers. Characterization of the catalytic activity will continue.

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