Cystine exodus from normal leucocytes is stimulated by MgATP

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Cystine exodus from partially purified granular fractions of normal leucocytes is stimulated by MgATP. *N*-Ethylmaleimide, an inhibitor of the lysosomal H⁺-translocating ATPase, eliminated the stimulated exodus, but had no effect on basal exodus. As the initial content of cystine was increased, the initial velocity of both the basal and ATP-stimulated egress increased. However, as saturation with substrate was approached, the ATP stimulation disappeared leaving only the *N*-ethylmaleimide-insensitive basal exodus. The increased initial velocity in the presence of ATP may represent improved binding of cystine to the partially saturated inner transporter as a result of conformational or charge optimization brought about by the action of the H⁺-translocating ATPase.

INTRODUCTION

ATP added to the incubation medium stimulates the exodus of cystine from granular fractions of lymphoblasts (Jonas *et al.*, 1982, 1983), fibroblasts (Smith *et al.*, 1987) and rat liver (Jonas, 1986). Gahl & Tietze (1985) have reported, however, that MgATP did not stimulate cystine exodus from granular fractions of human peripheral leucocytes. We have asked whether leucocytes, like the other tissues studied, would also show ATP-stimulated exodus under appropriate conditions.

EXPERIMENTAL

Leucocytes were prepared from freshly drawn heparinized blood as previously described (Greene *et al.*, 1985). Samples for normal data all came from the same donor. The freshly separated leucocytes were suspended in Hanks' balanced salt solution (containing calcium and magnesium) and incubated at 37 °C for 15–30 min in 0.15–1.0 mM-cystine dimethyl ester, adjusted to pH 7.2 with NaOH. After loading, a partially purified granular fraction was prepared as described by Jonas *et al.* (1983).

Cystine exodus was initiated by adding a 0.1 ml portion of granular fraction (40–75 µg of protein) to tubes containing 0.9 ml of buffer [20 mM-Hepes (pH 7.0)/0.25 M-sucrose/5 mM-MgCl₂] at 37 °C. ATP, neutralized to pH 7.0 with NaOH, was added 3 min after the start of the incubation to a final concentration of 2 mM. For some experiments (Table 1), 1 mM-NEM was also present from the start of incubation. The sample tubes were removed at intervals from 3 to 33 min, chilled in ice/water and centrifuged at 4 °C for 15 min at 20000 *g*. The pellet was suspended in 250 µl of 5 mM-NEM in phosphate buffer, pH 7.2, sonicated, and assayed for β-hexosaminidase, protein, and cystine as previously described by Jonas (1982). Cystine values are expressed as nmol of cystine/unit of β-hexosaminidase, a unit of hexosaminidase being defined as the amount able to hydrolyse 1 µmol of substrate/min. The integrity of the lysosomes during exodus remained unchanged, based on a constant value for units of β-hexosaminidase/mg of

protein (Lowry method). The percentage loss from the samples was compared with the percentage loss in other conditions by Student's *t* test.

RESULTS AND DISCUSSION

In three separate experiments normal leucocytes were loaded to levels ranging from 19 to 52 nmol of cystine/unit of β-hexosaminidase. A marked stimulation of cystine exodus from the granular fraction was observed in the presence of MgATP compared with the basal exodus observed with Mg²⁺ in buffer alone (*P* < 0.002). NEM prevented the stimulation, but had no effect on the basal exodus. When the cystine load was increased to 1200 nmol/hexosaminidase unit in an additional experiment, the effect was no longer apparent (Table 1).

A major difference between our experiments and those of Gahl & Tietze (1985) was the absence of NEM when we tested the effect of ATP. The method of Gahl &

Table 1. Cystine exodus from normal leucocyte granular fraction

Cystine exodus, measured as nmol of cystine/unit of β-hexosaminidase lost between 3 and 33 min, is given as a percentage of the 3 min value, mean ± s.d. (*n*). NEM, buffer (20 mM-Hepes/0.25 M-sucrose/5 mM-MgCl₂) and granular fraction were present from zero time; ATP was added at 3 min. Low load was 19–52 nmol of cystine/unit; high load was 1200 nmol/unit. The *P* values refer to differences between buffer (control condition) and each experimental condition of the low-load set.

	Low load		High load
	Loss (%)	<i>P</i>	Loss (%)
Buffer	54.5 ± 1.9 (3)	–	25.2 (1)
1 mM-NEM	51.6 (2)	0.40	29.8 (1)
2 mM-ATP	76.6 ± 4.8 (3)	< 0.002	23.6 (1)
2 mM-ATP + 1 mM-NEM	57.3 ± 8.1 (3)	0.56	20.1 (1)

Abbreviation used: NEM, *N*-ethylmaleimide.

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Fig. 1. Effect of initial cystine level on ATP stimulation of exodus

The initial velocity of exodus for different initial cystine levels (nmol/unit of β -hexosaminidase) was determined from the product of the initial cystine level and the first order rate constant derived by regression of $\ln$ (cystine/hexosaminidase) versus three to six time points (0–30 min). Bars represent an error for this product derived from the variance in determination of the load level and the standard error of the estimate for the rate constant (Arkin & Colton, 1950). Values for normal exodus represent leucocytes sampled from the same donor on eight different days ($\circ$, normal basal; $\bullet$, normal + ATP). On two occasions the sample was divided and loaded to different levels on the same day. Values for exodus from cystinotic leucocytes are from three different patients of which only the benign sample was preloaded as described for normals ($\square$, nephropathic cystinotic, basal; $\blacksquare$, + ATP; $\triangle$, benign cystinotic, basal; $\blacktriangle$, + ATP). Upper panel, higher load levels; lower panel, lower load levels with expanded scale.

Tietze (1985) requires the presence of NEM in order to show that loss of cystine from the lysosomes equals that which accumulates in the incubation medium. Without NEM, cysteine oxidation contributes to the external cystine. We have measured only the loss of intralysosomal cystine. Since NEM is an inhibitor of the H^+ -translocating ATPase (Flatmark *et al.*, 1982), the failure to show stimulation of cystine exodus in the presence of NEM could be explained by the requirement of a functioning ATPase for optimal cystine transport. The ATPase in turn is necessary for the maintenance of a transmembrane pH gradient. A positive correlation has been demonstrated between the stimulation of cystine exodus and ATP-dependent acidification in lymphoblasts (Jonas *et al.*, 1983), purified rat liver lysosomes (Jonas, 1986), and fibroblasts (Smith *et al.*, 1987). In these reports both exodus and acidification were blocked by inhibitors of the H^+ -translocating ATPase and agents which collapsed the transmembrane pH gradient.

Fig. 2. Cystine binding to lysosomal transporter

The Figure represents a lysosome with transmembrane H^+ -translocating ATPase (circle), cystine transporters (open polygons) and cystine (black polygons). The partially saturated transporter on the left provides for the basal exodus of cystine. The ATP-dependent entry of H^+ improves binding of cystine as shown with the transporter on the right, resulting in a stimulation of cystine exodus. When the H^+ entry is prevented by the inhibitor NEM, as indicated by the broken line, increased exodus is also prevented.

The disappearance of the ATP-dependent stimulation at high initial cystine levels led us to explore the relationship between exodus and load level. Saturation kinetics have been demonstrated for the basal exodus of cystine from leucocytes with NEM present (Gahl *et al.*, 1982). In our experiments without NEM, both basal and ATP-stimulated rates also showed an approach to saturation (Fig. 1). As would be expected of a saturable transporter, the time course of the low-load experiments was first-order, but that of the high-load experiments approached linear kinetics. The initial rate was stimulated 1.6–2.9-fold by addition of ATP when load levels were 800 nmol of cystine/hexosaminidase unit or less. At initial levels of 1200 nmol/unit and above, the rates of exodus with and without ATP were within experimental error of each other. Thus at the initial levels used by Gahl & Tietze (1985) no ATP stimulation would have been observed even in the absence of NEM.

In contrast with the characteristics of cystine exodus from normal leucocyte granules, granules prepared from leucocytes of patients with the lysosomal storage disease cystinosis showed minimal cystine loss with or without ATP. The percentage of initial cystine lost in 30 min from the granular fraction of three patients with cystinosis was 15, 0, and 3% of initial without ATP and 5, 9, and 0% with ATP, respectively. The initial cystine levels were 198, 174, and 116 nmol of cystine/unit of β -hexosaminidase.

Characterization of ATP stimulation of cystine exodus has shown: (1) that ATP fails to stimulate exodus when NEM is present, (2) that ATP stimulation is observed only at cystine load levels which are below saturation, and (3) that no ATP stimulation occurs in samples from patients with cystinosis.

Our interpretation of these results is that when the granules are isolated from leucocytes, the internal pH of the lysosomes is no longer optimal for the binding of

cystine to the cystine transporter. As illustrated in the speculative diagram in Fig. 2, the increase in exodus seen after the addition of ATP may be attributed to improved cystine binding to the partially saturated transporter. Such an improved binding may be the result of conformational or charge optimization brought about by the entry of H^+ via the H^+ -translocating ATPase. Thus H^+ could be considered a positive allosteric modifier for the binding of cystine to the transport system on the inner membrane surface. A proper membrane potential maintained by ATP as observed in fibroblasts (Smith *et al.*, 1987) may also play a role. At high load levels, however, the carrier is saturated and no further improvement in binding of substrate is provided by pH and charge optimization. It follows that under these conditions neither ATP nor NEM would have any effect. In contrast with the normal pattern, leucocyte lysosomes of cystinotic patients showed no exodus and no response to ATP regardless of initial level, a finding attributable to a defective transporter.

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