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DRUG INHIBITABLE ECTO-ATPase IN LEUKOCYTES

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SUMMARY

In rat leukocytes, the total ATP-hydrolyzing activity was highest when determined in cells with an intact plasma membrane. The ecto-ATPase, exhibiting a Km for ATP of 0.3 mM, was inhibited 30% by sulfhydryl reagents, whereas dinitrophenol, oligomycin or Ca^{2+} had little or no effect. The ouabain inhibitable, Na, K-activated component amounted to 7% of the total ATPase activity in leukocytes. A major characteristic of the ecto-ATPase was its marked inhibition by micromolar concentrations of both phenothiazine tranquilizers and tricyclic antidepressants.

The total cellular ATP-hydrolyzing activity represents a variety of, more or less well characterized, specific ATPase components. In addition to the Na,K-ATPase, the enzyme involved in the transmembrane transport of monovalent cations (1), numerous Mg-activated ATPases, linked to specific biological processes were reported (e.g., 2-4). Despite the diversity in structure and biological role, a common characteristic of the ATPases is their particulate nature. In addition to being membrane-bound, most ATPases recognize only intracellular ATP as their substrate. Reports on the presence of ecto-ATPases, localized on the outside of the plasma membrane of cells, are few (5-8). In the course of our studies

on the interaction of CNS drugs with components of cell membranes (9-11), we recently obtained evidence for the existence of a drug inhibitable ecto-ATPase in rat leukocytes. The present work provides data on the localization and characterization of this enzymatic activity.

MATERIALS AND METHODS

Materials. Desipramine, nortriptyline, trifluoperazine and thioridazine were gifts from Drs. E. F. Domino and H. H. Swain, Department of Pharmacology, The University of Michigan. The biochemicals and enzymes were purchased from Sigma Chemical Co., St. Louis, Mo. and from Boehringer-Mannheim Co., New York, N. Y. Plasmagel, a modified gelatin solution, was obtained from HTI Corporation, Buffalo, N. Y. All other chemicals were of reagent grade.

Isolation of leukocytes. These cells were isolated from blood of 300 g male, Sprague-Dawley rats as described previously (12). Briefly, erythrocytes were separated from the other cells by sedimentation in the presence of Plasmagel. Leukocytes were further separated from platelets by differential centrifugation and obtained within 1.5 hr after the collection of blood. The purity and viability of the isolated cells were investigated in detail and ascertained (12). Cellular viability was evaluated by the cellular contents of K^+, Na^+ and ATP, as well as by the uptakes of trypan blue and O_2.

Treatment of biologic material. The isolated leukocytes were suspended in a buffer medium containing the following concentrations (mM): Tris. HCl, 35; NaCl, 120; KCl, 5; $MgCl_2$, 2.5 and glucose, 10. The pH of the medium was adjusted to 7.4. Aliquots of the above suspension were subjected to the

assay of enzymatic activity as such, or after one of the following pretreatments: disruption in an all-glass Potter-Elvehjem homogenizer, repeated freezing at -70° and thawing, or, sonication for 3 x 20 sec at maximal output of a standard microtip, Branson Sonifier (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.).

Assay of ATPase activity. The enzymatic activity was quantitated as described (11) by colorimetric or enzymatic determination of inorganic phosphate released from ATP during incubation at 37°. The computed tonicity of the assay medium was 300.5 mosmol.

Protein was estimated according to Lowry *et al.* (13).

Determination of K^+ and Na^+. As a measure of an intact plasma membrane, the cellular concentrations of the above ions were determined by flame photometry as described previously (9,12). Briefly, aliquots of the cellular suspension were centrifuged and thoroughly washed by repeated resuspension with ice-cold isotonic sucrose, adjusted to pH 7 with Tris base. Subsequently, the cellular pellet was digested with conc. HNO_3, diluted with 15 mM LiCl and subjected to analysis by flame photometry.

RESULTS

In freshly isolated rat leukocytes, the total ATP-hydrolyzing activity averaged to 11 ± 0.9 μmoles Pi/mg protein/hr. Optimum activity was obtained in the pH range of 7.0 to 7.8. A double reciprocal plot of the relationship of enzyme activity and substrate concentration yielded a Km of 0.3 mM (Fig.1). The ATPase was inhibited 7% by ouabain, 30% by sulfhydryl reagents (except iodoacetamide) and about 10% by DNP (Table 1).

TABLE 1

Inhibition of ATPase activity in leukocytes by various agents

Compound and its concentration (mM)		ATPase activity in intact cells (% of control)
Ouabain	1	93.3
PCMB	1	68.8
PHMB	1	68.6
NEM	1	72.0
IA	1	99.1
OM (μg/ml)	67	95.5
DNP	3	89.4
Ca^{2+}	5	99.0
Desipramine	0.5	30.6
Nortriptyline	0.5	13.3
Trifluoperazine	0.15	5.0
Thioridazine	0.15	1.0

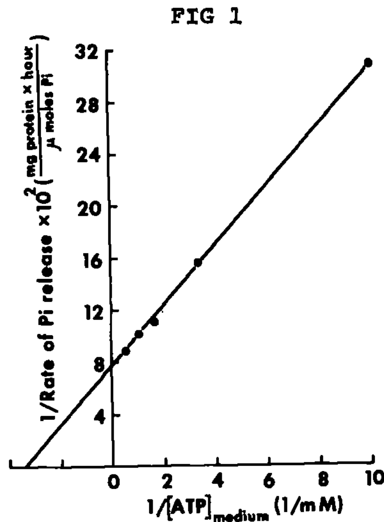
Suspensions of intact leukocytes were incubated for 15 min at 37° with the listed compounds present at concentrations as indicated. Subsequently, ATP was added and the incubation continued for 30 min. The further treatment of the cellular preparation and the estimation of Pi was carried out as described under Materials and Methods. Presented are mean values of results obtained in 4 experiments using different preparations of leukocytes. Abbreviations: PCMB, p-chloromercuribenzoic acid; PHMB, p-hydroxymercuribenzoic acid; OM, olygomycin, DNP, 2,4-dinitrophenol; NEM, n-ethyl maleimide; IA, iodoacetamide.

Oligomycin or Ca^{2+} had little or virtually no effect on the enzymatic activity.

However, the ATPase was markedly inhibited by both tricyclic antidepressants and phenothiazine tranquilizers (Table 1). A detailed investigation of the latter interactions was presented elsewhere (11).

Maximum ATPase activity in leukocytes was consistently obtained with cells which after isolation were suspended in isotonic buffer medium, without any additional treatment.

FIG 1



Lineweaver-Burk plot of the enzymatic Pi release from ATP by intact leukocytes. Cells were incubated with varying concentrations of ATP for 40 min at 37°. Released Pi and protein were determined as described in the text. The colorimetric estimation of Pi was utilized. Presented are the results of a typical experiment.

Disruption of the plasma membrane by various means resulted in decreased ATPase activity (Table 2). In order to ascertain the integrity of the cells during the ATPase assay, the contents of K^+ and Na^+ in leukocytes were determined at various times during the incubation. The results of these experiments showed a high ratio of cellular K^+ to cell Na^+ throughout the enzyme assay (Table 2).

The possibility that the observed release of Pi was a consequence of phsophatase activity, was investigated by varying the conditions under which the ATPase assay was carried out (Table 3). ATPase activity was identical both in the presence and absence of glucose in the incubation medium. Replacing ATP by glucose 1- or 6-phosphate, or omitting Mg^{2+}, resulted in loss of enzymatic activity. In these experiments,

TABLE 2

ATPase activity and ionic content in leukocytes after their exposure to various conditions.

Conditions of pretreatment	ATPase activity (μg Pi/mg protein/hr)	Cell content of K^+ (nEq/mg protein)	Na^+
Incubation, 5 min, 37°	361±17	520	51
Incubation, 40 min, 37°	357±14	483	54
Freeze-thawed	180±5	-	-
Homogenized	297±10	-	-
Freeze-thawed and homogenized	197±13	-	-
Sonified	240±15	-	-

In suspensions of freshly isolated rat leukocytes, ATPase activity was determined as described in the text. In addition, at 5 min and 40 min of the assay the cellular contents of K^+ and Na^+ were estimated by flame photometry. In other experiments, prior to the ATPase assay, the cells were initially subjected to the various treatments indicated. Presented are mean values and the standard deviation of 6 individual measurements.

similar results were obtained using either intact or disrupted cells.

DISCUSSION

The total ATP-hydrolyzing activity in leukocytes was maximal when estimated in intact cells. The presence of non-leaking plasma membranes was ascertained by the high ratio of cellular K^+/Na^+, determined in leukocytes at different times during the ATPase assay. Purposeful disruption of the cells by various means resulted in decreased enzyme activity. The recognition of extracellular ATP by leukocytes suggest the existence of an ecto-ATPase in these cells. The ecto-ATPase in leukocytes was partially affected by SH-group inhibitors, however the maximum effect, obtained with PHMB or PCMB, was only moderate relative to the potent inhibition of the enzyme

TABLE 3

Effect of composition of incubation medium on ATPase activity in leukocytes

Specific changes in medium composition	ATPase activity (% of control)
Intact cells:	
+ glucose, + ATP	100
+ glucose, - ATP	<1
- glucose, + ATP	101
- glucose, - Mg^{2+}, + ATP	2
- glucose, - ATP, + gl-p	<1
- glucose, - ATP, + g6-p	<1
Disrupted cells:	
+ glucose, + ATP	100
+ glucose, - ATP	1
- glucose, + ATP	102
- glucose, - Mg^{2+}, + ATP	5
- glucose, - ATP, + gl-p	2
- glucose, - ATP, + g6-p	1

Suspensions of intact and disrupted leukocytes were incubated at pH 7.2 in the ATPase assay medium described under Materials and Methods but modified by the addition and omission of various components as indicated. After 30 min at 37°, the Pi content of the medium was determined spectrophotometrically at 700 nm as described in the text. Presented are mean values of results obtained in 3 experiments. Abbreviation: gl-p, glucose l-phosphate; g6-p, glucose 6-phosphate.

activity by phenothiazine tranquilizers and tricyclic antidepressants.

An interference of phosphatase in the characterization of the ecto-ATPase was excluded. No phosphatase activity at neutral pH was detected in either intact or in disrupted leukocytes. In view of the considerable role of glycolysis in the metabolism of the latter cells (14), phosphate esters of glucose were utilized as substrates for possible phosphatase activity. At pH 9.9, glucose 1-phosphatase activity in leukocytes has been demonstrated (15).

Ecto-ATPase activity was reported previously in glial cells dissected from rabbit brain and a biologic role for such an enzyme discussed (6). Evidence has been presented for the existence of an ATPase localized on the external surface of platelets (7,16,17), and a positive correlation between the activity of the ecto-ATPase and platelet aggregation was described (17). Unfortunately, these studies failed to provide data on the morphologic state of the investigated "intact" platelets, i.e., to confirm the presence of a nonleaking plasma membrane.

In leukocytes isolated from acute peritoneal exudates of the rabbit, as well as in form of the buffy coat from rabbit and human blood, an ATPase activity was reported in undisrupted cells (5). Although, again, no evidence was presented on the condition of the plasma membrane of the cells, the reported enzymatic activity had some characteristics similar to the ones described in the here presented work. The similarities, in addition to the ecto-nature of the ATPase, were: total dependence on Mg^{2+}, a 30-35% inhibition by 5 mM NEM and a pH optimum of 7.4. However, a number of differences exist. The ATPase activity in leukocytes from the buffy coat was inhibited 50% by ouabain, a value which is much higher than both our and previously reported data for leukocyte ATPase. Furthermore, Ca^{2+} inhibited the ATPase in leukocytes from both, peritoneal exudates and from the buffy coat. In addition to the lacking data on the viability of the isolated cells, essential for the confirmation of an ecto-enzyme, no information was provided regarding the purity of the leukocytes. A possible contamination of these cells could have been a contributing factor to some of the marked differences between the data reported

in that paper (5) and the results presented here. Recently, an ecto-ATPase was described in guinea pig polymorphonuclear leukocytes (8). Except for ouabain, which had no effect, the inhibition pattern obtained with sulfhydryl reagents, DNP and oligomycin was quite similar to the one observed in the present work (18). However, the Km of the enzyme in guinea pig leukocytes was about ten times lower then the corresponding value determined in rat leukocytes (Fig. 1). In that study (8), evidence was also presented for an adenosine monophosphatase and a p-nitrophenyl phosphatase, both ecto-localized in guinea pig leukocytes.

In addition to the species difference, a possible factor contributing to the differences in the properties of the hitherto reported ecto-ATPases in leukocytes (5,8,18) is the heterogeneity of the utilized cells. It should however be emphasized that in neither of the above cited reports was evidence presented for the marked drug-enzyme interactions, which characterized the enzymatic activity described in the present study.

The biologic role for an ecto-ATPase in leukocytes is yet unknown. The recognition of extracellular ATP raises the question of availability of this substrate in that compartment *in vivo*. The function of an ecto-ATPase could be expected to be linked to energy-requiring processes, as represented by the pronounced surface activity of leukocytes, including their phagocytic activity as well as immunologic function. Evidence for a correlation of surface phenomena in a circulating blood cell with the activity of an ecto-ATPase has been established in case of platelet aggregation (17). The most impressive characteristics of the here described ecto-ATPase in leukocytes

was its potent inhibition by phenothiazine tranquilizers and tricyclic antidepressants, unmatched by a variety of other CNS drugs (11). Assuming a link between the surface activity of leukocytes and their ecto-ATPase, an inhibition of the latter by drugs could lead to an impaired cellular defense system of patients receiving these compounds.

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