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EFFECTS OF *N*-ACETYLIMIDAZOLE ON HUMAN ERYTHROCYTE ATPase ACTIVITY

EVIDENCE FOR A TYROSYL RESIDUE AT THE ATP BINDING SITE OF THE $(Na^+ + K^+)$-DEPENDENT ATPase

STANLEY J. MASIAK and GUY D'ANGELO

Department of Physiology and Biophysics, Health Sciences Center, State University of New York, Stony Brook, N.Y. 11794 (U.S.A.)

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SUMMARY

1. Acetylation of human erythrocytes by *N*-acetylimidazole alters the structure of stroma prepared from these cells and the degree of alteration appears to be dependent upon the level of the initial treatment. These changes do not occur when stroma are acetylated.

2. Deacetylation by hydroxylamine or mild alkaline treatment causes a complete recovery of the $(Na^+ + K^+)$-dependent and the Ca^{2+}-stimulated ATPase activities and indicates that the inhibition is due to the acetylation of a tyrosyl residue. There is only partial recovery of the Mg^{2+}-dependent ATPase after deacetylation.

3. ATP or Mg–ATP completely protect the $(Na^+ + K^+)$-dependent ATPase, but not the Ca^{2+}-stimulated system.

4. The results indicate that the $(Na^+ + K^+)$-dependent and the Ca^{2+}-stimulated ATPase activities have separate substrate binding sites and most likely are separate enzyme systems.

5. Acetylation of human erythrocytes has no effect on D-glucose transport.

INTRODUCTION

Protein-modifying agents are currently being used for the identification of membrane components located at the external surface of the erythrocyte membrane [1–4]. From these and other studies, it is agreed that only a fraction of the membrane material, especially protein, is accessible to the modifying agents from the exterior surface of the cell [5]. Recently, procedures have been developed for the labelling of interior surface proteins [6, 7]. However, very little is known about the effects of the exterior surface treatment on the alteration of availability of reactive sites of membrane components at the interior surface of the cell.

The uptake of K^+ and the extrusion of Na^+ from human erythrocytes is an active process which uses Mg–ATP as a direct energy source [8]. The extrusion

of Ca^{2+} from the human erythrocyte is also an active process, requiring Mg–ATP as its energy source [9]. These transport systems appear in isolated red cell stroma as $(Na^+ + K^+)$-dependent and Ca^{2+}-stimulated ATPase systems, both requiring the presence of Mg^{2+} [9–12]. In addition, the erythrocyte membrane contains a Mg^{2+}-dependent ATPase which may or may not be part of the transport systems [8].

The purpose of the present study with *N*-acetylimidazole, an acetylating agent, is several fold: (a) to determine if there is an alteration in membrane structure after exterior cell treatment, using changes in the membrane ATPase activity as an index, (b) to use the specificity of the agent to determine the residue involved in ligand binding (*N*-acetylimidazole is quite specific for tyrosyl residues under the proper experimental conditions [13, 14]), and (c) to determine if the presence of the various ligands during the acetylation procedure will prevent inhibition.

MATERIALS AND METHODS

Preparation of erythrocyte stroma

Human erythrocytes were obtained from outdated blood in standard acid–citrate–dextrose bags from the Nassau-Suffolk (N. Y.) Inter-County Blood Services, a division of the Greater New York Blood Program. Erythrocyte stroma were prepared according to the method of Masiak and Green [15]. The final wash of the membrane material was in distilled water to remove essentially all the Tris buffer. The protein content of each membrane preparation was determined by the method of Lowry et al. [16] with trypsin, 2X crystalline, salt-free (Worthington Biochemical) as standard.

ATPase assay

ATPase activity was assayed by measuring the inorganic phosphate released from ATP during 1 h incubation at 37 °C. Phosphate was measured using a modification of the method of Fiske and SubbaRow [17]. Incubations were in a volume of 3 ml with 2 ml removed for actual assay. All tubes usually contained 1.5 mM Tris–ATP, 1.5 mM Mg^{2+}, 120 mM Na^+ and 45 mM Tris–HCl buffer (pH 7.4). All additions and modifications are given in the text. All cations were added as their chloride salts. Disodium ATP was obtained from Sigma Chemical Co., St. Louis, Mo. ATP solutions were passed through columns of Dowex 50W-8X in the H-form to remove sodium and trace amounts of calcium. The solutions were immediately adjusted with 45 mM Tris buffer (pH 7.4) and stored at −20 °C until used.

Mg^{2+}-dependent ATPase activity is the activity measured in the presence of only Mg^{2+} and Na^+ or in the presence of Mg^{2+}, Na^+, K^+ and ouabain. The $(Na^+ + K^+)$-dependent portion of the activity is calculated by subtracting the Mg^{2+}-dependent activity from the total activity in the presence of Mg^{2+}, Na^+, and K^+. The Ca^{2+}-dependent portion of the activity is calculated by subtracting the Mg^{2+}-dependent activity from the activity in the presence of Mg^{2+} and Ca^{2+}. The Na^+-dependent portion of the Ca^{2+}-stimulated activity is calculated by subtracting the Ca^{2+}-stimulated activity from the activity in the presence of Mg^{2+}, Ca^{2+} and Na^+.

Acetylation of stroma

The stroma (20 %, v/v) were treated with *N*-acetylimidazole (Pierce Chemical Co.) in 10 mM Tris buffer (pH 7.4) for 1 h at room temperature. The stroma and *N*-acetylimidazole were always in a mgm:mgm ratio, except where noted. The pH of the reaction remained essentially constant during the treatment period. The suspension was centrifuged for 15 min at 30 000 $\times g$. The treated stroma were washed with distilled water to remove any unreacted *N*-acetylimidazole. Protein determinations were done on the acetylated stroma by the method of Lowry et al. [16]. In all instances, control membrane material was treated identically except for the omission of the *N*-acetylimidazole.

The acetylated membrane material was occasionally treated a second time with the *N*-acetylimidazole. This treatment was done in the manner described above. Protein determinations were always done on the retreated preparations.

Acetylation of intact cells

The reactions were run on outdated human blood. The cells were washed 4 times in 140 mM NaCl and 10 mM Tris buffer (pH 7.4) and the buffy coat removed by aspiration. The washed cells were warmed to room temperature and the *N*-acetylimidazole was added. The reaction mixture (20 % cells, v/v) was gently mixed for 1 h at room temperature. The treated cells were washed 3 times with 0.15 M Tris buffer (pH 7.0) at 4 °C. A second treatment of this membrane preparation with *N*-acetylimidazole was done as described above.

Diazotization of stroma

The diazonium salt of sulfanilic acid was prepared immediately before use by the indirect method [18] and washed once with a small amount of cold water. The stroma were treated as described by Masiak and D'Angelo [19] usually with a final concentration of 48 μM. Protein determinations were done on the diazotized stroma by the method of Lowry et al. [16].

Diazotized stroma were treated with *N*-acetylimidazole, as described above. In some instances, acetylated stroma were treated with the diazonium salt of sulfanilic acid. Protein determinations were always done before the stroma ATPase activity was determined. Control preparations were always treated identically, except for the omission of the modifying agent.

RESULTS

Treatment of human erythrocytes with a low level of *N*-acetylimidazole causes a 25–35 % inhibition of the various ATPase activities (Table I). When the agent is increased 4-fold, there is at most a 10 % increase in inhibition, even though it should penetrate the red cell membrane with ease, as has been shown for another acetylating agent, acetic anhydride [20]. It should be noted that the amount of inhibition does not change with time. This is in direct contrast to the observed effects when red cell ghosts are treated (see Fig. 1).

When stroma from the above experiments are retreated with a fixed amount of *N*-acetylimidazole, there is not a fixed increase in the amount of inhibition for each type of ATPase activity (Table II). The Mg^{2+}-dependent ATPase is inhibited

TABLE I

STROMA ATPase ACTIVITY AFTER WHOLE CELL TREATMENT WITH *N*-ACETYLIMIDAZOLE

Erythrocytes were acetylated with *N*-acetylimidazole as described in Methods. The data were expressed as μM P_i/mg protein per h and are averages of three determinations which were within 5 % of each other.

N-Acetylimidazole/ml packed cells (mg)	Mg^{2+}-dependent ATPase	$(Na^+ + K^+)$-dependent ATPase	Ca^{2+}-stimulated ATPase
None	0.175	0.370	1.038
2.5	0.130	0.235	0.723
5.0	0.120	0.230	0.704
10.0	0.112	0.213	0.717

to the same level as the untreated control (approx. 60 % for a single treatment of stroma and for a combined whole cell and stroma treatment). The $(Na^+ + K^+)$-dependent ATPase shows only a minimal increase in inhibition and is not as inhibited as a single treatment of stroma. The Ca^{2+}-stimulated ATPase is markedly reduced upon retreatment and the amount of inhibition is dependent upon the initial treatment

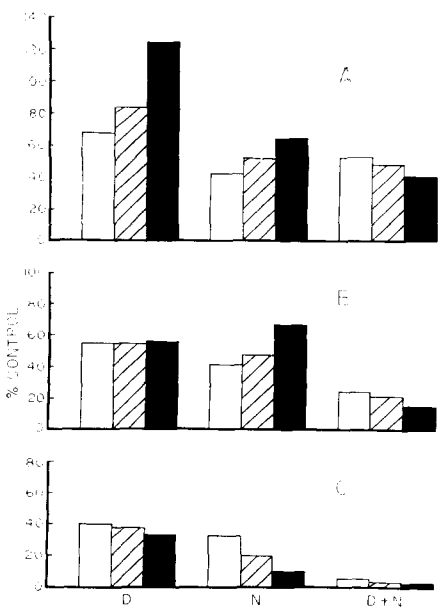

Fig. 1. Individual and combined effects of diazotization and acetylation on stroma ATPase. Stroma were treated with 48 μM diazonium salt of sulfanilic acid (D) and with 1 mg *N*-acetylimidazole per mg protein (N) as described in Methods. In the dual treatment experiments (D+N), stroma were treated with the diazonium salt and immediately retreated with *N*-acetylimidazole as described in Methods. Control preparations were treated identically, except for the omission of the modifying agent. The stroma were assayed for ATPase activity at various time intervals after treatment: 1 h, clear bar; 1 day, cross-hatched bar; 4 days, solid bar. A, $(Na^+ + K^+)$-dependent ATPase; B, Mg^{2+}-dependent ATPase; C, Ca^{2+}-stimulated ATPase.

TABLE II

REACETYLATION OF WHOLE CELL-TREATED STROMA

Stroma, prepared after whole cell acetylation (Table I), were treated a second time with 1.0 mg *N*-acetylimidazole/mg protein as described in Methods. The data are expressed as μM P_i/mg protein per h and are averages of three determinations which were within 5 % of each other.

N-Acetylimidazole/ml packed cells (mg)	Mg^{2+}-dependent ATPase	$(Na^+ + K^+)$-dependent ATPase	Ca^{2+}-stimulated ATPase
Unmodified Stroma	0.175	0.370	1.038
None	0.066	0.156	0.354
2.5	0.064	0.221	0.295
5.0	0.065	0.192	0.175
10.0	0.059	0.190	0.054

of the whole cells (compare Table II with Table I). The stroma, prepared from acetylated whole cells, apparently have a partially altered structure and the degree of alteration is dependent upon the level of the whole cell treatment.

Table III shows the effects of increasing acetylation on the various ATPase activities from stroma, prepared from untreated whole cells. There is a progressive increase in the inhibition of the ATPase activities with an increase in *N*-acetylimidazole levels, with the Ca^{2+}-stimulated ATPase most sensitive to this treatment. This is in marked contrast to the acetylation of whole cells (see Table I). If these preparations are retreated immediately with a fixed amount of *N*-acetylimidazole (0.5 mg/mg protein), the amount of added inhibition is constant, irrespective of the initial treatment. Stroma, prepared from untreated whole cells, apparently do not undergo a structural alteration upon acetylation. These results also suggest that more care must be used in interpretation of studies of membrane structure which use dual labeling procedures (exterior surface labeling followed by membrane fragment labeling) to determine the locations of membrane components, since some alteration in availability of reactive sites on the interior surface may occur after exterior surface treatment.

Acetylation of sulfhydryl groups is not observed because the products immediately hydrolyse in aqueous solutions [21]. However, both amino and tyrosyl

TABLE III

INHIBITION OF STROMA ATPase WITH INCREASING ACETYLATION

Erythrocyte stroma were treated as described in Methods. The data are expressed as % control activity and are averages of three determinations which were within 5 % of each other.

N-Acetylimidazole/ mg protein (mg)	Mg^{2+}-dependent ATPase	$(Na^+ + K^+)$-dependent ATPase	Ca^{2+}-stimulated ATPase
0.25	87.0	88.3	74.4
0.50	82.0	53.6	52.0
0.75	61.0	46.1	42.6
1.00	42.0	43.8	33.9
2.00	36.0	22.6	4.5
3.00	6.7	3.9	2.1

TABLE IV

RECOVERY OF ATPase ACTIVITY BY DEACETYLATION

Stroma were treated with 1.0 mg *N*-acetylimidazole/mg protein and were deacetylated with hydroxylamine according to the method of Balls and Wood [22]. Control preparations were treated identically. The data are expressed as % of the appropriate control activity and are averages of four determinations which were within 3 % of each other.

Deacetylation procedure	Mg^{2+}-dependent ATPase	$(Na^+ + K^+)$-dependent ATPase	Ca^{2+}-stimulated ATPase
None	42.0	43.8	33.9
Hydroxylamine	68.4	93.8	95.6
pH 8.1, 1 h	63.9	74.9	55.4
pH 8.1, 4 h	60.5	91.3	89.1

residues can be acetylated and are distinguishable by the ease with which the latter can be reversed. Hydroxylamine at neutral pH or a mild alkaline treatment are sufficient to deacetylate tyrosyl residues [22], but not amino groups which require hot acid or base [21]. The $(Na^+ + K^+)$-dependent and Ca^{2+}-stimulated ATPase completely recover after deacetylation with hydroxylamine (Table IV). Treatment of the membrane preparations at pH 8.1 for 4 h also gives nearly complete recovery. For these two ATPase systems, it appears that the inhibition is due to the acetylation of tyrosyl residues. The Mg^{2+}-dependent ATPase shows only partial recovery.

The effects of *N*-acetylimidazole with various ligands present in the reaction mixture are shown in Table V. The ATPase activities were assayed within 1 h after

TABLE V

EFFECTS OF VARIOUS LIGANDS ON *N*-ACETYLIMIDAZOLE INHIBITION OF STROMA ATPase ACTIVITY

Stroma were treated with 1.0 mg *N*-acetylimidazole/mg protein in the presence of the indicated ligand as described in Methods. Control preparations were treated identically, except for the omission of *N*-acetylimidazole. The data are expressed as % of the controls.

Ligand	Mg^{2+}-dependent ATPase	$(Na^+ + K^+)$-dependent ATPase	Ca^{2+}-stimulated ATPase
None	42.0±1.4 ($N = 6$)	43.8±0.8 ($N = 6$)	33.9±1.7 ($N = 6$)
3 mM ATP	65.1±2.1	95.6±1.3	31.1±1.6
3 mM ATP + 3 mM Mg^{2+}	100±4.1	92.3±1.5	52.0±0.7
3 mM ATP + 3 mM Mg^{2+} + 120 mM Na^+	78.7±1.6	93.5±1.7	70.3±1.1
3 mM Mg^{2+}	43.41±1.3	25.1±3.21	52.1±0.7
120 mM Na^+	56.2±2.1	30.1±1.3	41.4±0.3
20 mM K^+	43.6±4.1	30.5±1.2	36.1±1.5
0.05 mM Ca^{2+}	35.6±0.4	41.5±3.2	28.5±2.1

treatment. ATP or Mg–ATP completely protect the $(Na^+ + K^+)$-dependent ATPase, but not the Ca^{2+}-stimulated system. The Mg^{2+}-dependent ATPase is partially protected by ATP alone and completely protected by Mg–ATP. It is impossible to state at this time whether the protected tyrosyl residues for the $(Na^+ + K^+)$-dependent and Mg^{2+}-dependent systems are identical.

When Na^+, K^+ or Mg^{2+} are present during acetylation, there is an enhanced inhibition of the $(Na^+ + K^+)$-dependent ATPase, but not of the Mg^{2+}-dependent or Ca^{2+}-stimulated systems. These results can be interpreted as a relatively specific conformational change enhancing the reactivity or increasing the number of available sites for acetylation of the $(Na^+ + K^+)$-dependent ATPase. However, it is impossible to state this with certainty since similar observations might be obtained by non-specific membrane alteration. A quantitative determination of the number of tyrosyl residues, acetylated in the presence of ions or protected in the presence of nucleotides, was attempted using the method of Balls and Wood [22], but the method is not sensitive enough to distinguish small changes in acetylation.

Fig. 1 shows the individual and combined effects of diazotization and acetylation of the various ATPase activities. The effects of diazotization are the same as those previously reported [19]. The $(Na^+ + K^+)$-dependent and the Mg^{2+}-dependent ATPase from acetylated stroma recover with time, but the Ca^{2+}-stimulated ATPase becomes more inhibited with time. Proteolysis of the membranes is not the cause of these changes since the electrophoretic patterns (0.1 % sodium dodecylsulfate, 8 M urea, 5 % acrylamide gels [23]) were unchanged over the entire experimental period. It should be noted that there was no change in inhibition of any of the ATPase activities over a similar time period for stroma prepared from acetylated whole cells.

The coupling reaction of the diazonium salt of sulfanilic acid is pH sensitive with the optimum for phenolic groups about pH 7.5 [24] but at an acidic pH the diazonium salt will show a greater preference for amine groups [25]. Unfortunately, both reactions occur over the entire pH range. In an attempt to determine which group is involved, stroma were treated first with the diazonium salt and then acetylated. Since the inhibition of the $(Na^+ + K^+)$-dependent ATPase is not additive upon dual treatment, it appears likely that the diazonium salt reacts with the same tyrosyl residues as *N*-acetylimidazole. When reciprocal experiments were done, similar results were obtained. The $(Na^+ + K^+)$-dependent ATPase no longer recovers with time after a dual treatment. Since the inhibition of the Ca^{2+}-stimulated and the Mg^{2+}-dependent activities are partially additive, it appears likely that both phenolic and amine groups are involved in the inhibition.

Human erythrocytes were treated with 2–10 mg *N*-acetylimidazole for up to 2 h and the rate of D-glucose movement was determined densitometrically as described by LeFevre [26]. There was no inhibition of D-glucose transport by acetylation of whole cells.

DISCUSSION

While acetylation is not limited to a specific residue, the proper deacetylation conditions can give some insight into the group acetylated. Since hydroxylamine or mild alkaline treatment causes complete recovery of the $(Na^+ + K^+)$-dependent

and the Ca^{2+}-stimulated ATPase systems, the acetylation of a tyrosyl residue appears responsible for the inhibition of both systems. Since ATP or Mg–ATP can protect against inhibition of the $(Na^+ + K^+)$-dependent, but not the Ca^{2+}-stimulated ATPase, it is reasonable to conclude that a tyrosyl residue is located at the site of ATP binding for the $(Na^+ + K^+)$-dependent ATPase and that a different tyrosyl residue, not located at its substrate binding site, is involved in the inhibition of the Ca^{2+}-stimulated ATPase. This would indicate, but not necessarily prove, that the substrate binding sites for each enzyme are separate, and that each ATPase activity represents an independent enzyme system. The latter statement is in agreement with tentative conclusions from studies of the phosphorylated intermediates of both ATPase activities [27].

Since there is only a partial recovery of the Mg^{2+}-dependent ATPase and a partial protection by ATP, both an amine group and a tyrosyl residue may be involved in the inhibition. Unfortunately, an irreversible structural alteration during the modification procedure would also give the same results, and at the present time, it is impossible to distinguish the two. Even though the protection patterns for the Mg^{2+}-dependent and $(Na^+ + K^+)$-dependent ATPase systems are different, it is still impossible to state that the substrate binding sites are separate. However, the Mg^{2+}-dependent ATPase does appear to have a separate substrate binding site from the Ca^{2+}-stimulated activity.

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