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EFFECTS OF CATIONS ON THE ADENOSINE DIPHOSPHATE–ADENOSINE TRIPHOSPHATE EXCHANGE REACTION CATALYZED BY RAT BRAIN MICROSOMES

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SUMMARY

Some properties of a $(Na^+ + Mg^{2+})$-dependent ADP–ATP exchange reaction catalyzed by NaI-treated rat brain microsomes have been investigated. The $(Na^+ + Mg^{2+})$-activated exchange exhibited an absolute requirement for Na^+, was inhibited by ouabain and stimulated 2–3-fold by oligomycin. The optimum $MgCl_2$ concentration for the $(Na^+ + Mg^{2+})$-activated exchange was 1.0 mM, but in the presence of oligomycin 3.0 mM $MgCl_2$ was required for optimum activity. The exchange reaction exhibited a complex multiphasic response to increasing concentration of Na^+, with activity increasing up to 200 mM NaCl.

INTRODUCTION

The coupled transport of Na^+ and K^+ across cellular membranes is closely related to the activity of membrane bound $(Na^+ + K^+)$-ATPase[1–3]. The precise nature of the relationship between $(Na^+ + K^+)$-ATPase and ion transport, however, is still not clear. The reaction sequence of the $(Na^+ + K^+)$-ATPase has been investigated extensively in an effort to elucidate the role of this enzyme complex in the Na^+ and K^+ active transport process. Certain $(Na^+ + K^+)$-ATPase preparations also have been demonstrated to catalyze a Na^+-dependent ADP–ATP exchange reaction[4–10]. On the basis of these and other results it has been postulated that the initial step in the $(Na^+ + K^+)$-ATPase reaction is a Na^+-dependent phosphorylation of the enzyme[3].

In this paper we report on the relationship between NaCl concentration and the activity of a Na^+-dependent ADP–ATP exchange reaction catalyzed by NaI-treated rat brain microsomes possessing an active $(Na^+ + K^+)$-ATPase. The Na^+-dependent exchange reaction was optimal in the presence of 1.0 to 3.0 mM $MgCl_2$ and exhibited a complicated multiphasic response to increasing concentrations of NaCl. The complex response to increasing NaCl is of interest because salt uptake by certain tissues exhibits multiphasic responses to increasing salt concentrations[11–13].

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EXPERIMENTAL

Materials

Ouabain, oligomycin, sodium deoxycholate and the Tris salts of ATP and ADP were obtained from Sigma Chemical Co. St. Louis, Mo. U.S.A. [U-^{14}C]ADP (231 Ci/mole) and [8-^{14}C]ADP (39 or 43 Ci/mole) were obtained from Schwarz Bio-Research Inc., Orangeburg, N.Y., U.S.A., as the tri-lithium salts in a 50% v/v ethanolic solution. Before use, the labelled ADP solutions were evaporated to dryness in a stream of N_2 and then redissolved in an aqueous solution of unlabelled ADP. Polyethyleneimine cellulose for thin-layer chromatography was purchased from Nutritional Biochemicals, Cleveland, Ohio, U.S.A., BioRad Laboratories, Richmond, Calif. U.S.A., or Gallard Schlesinger Chemical Manufacturing Corp. Carle Place, L.I., N.Y., U.S.A. All other reagents were of the best quality available from commercial sources. Fresh frozen rat brains were obtained from Miles Laboratories, Kankakee, Ill., U.S.A., and stored at -80 °C.

Preparation of rat brain microsomes

Microsomes were prepared from rat brains and treated with NaI according to the procedures of Akera and Brody[14,15]. The $(Na^+ + K^+)$-ATPase activity of these preparations varied between 130 and 220 μmoles of ATP hydrolyzed/h per mg protein. This activity, at pH 7.8, accounted for 97% to 99% of the total ATPase activity in the microsomes.

Protein determination

After dispersing the microsomes in 5% sodium deoxycholate, protein was determined according to the method of Lowry *et al.*[16] with bovine serum albumin as the standard.

Assay of ADP–ATP exchange activity

The assay of exchange activity was performed according to the procedures of Fahn *et al.*[4]. The standard reaction mixture in a final volume of 50 μl contained: 5.0 mM Tris–ATP, 1.2 mM Tris [^{14}C]ADP (55500 or 111000 dpm), 1.0 mM $MgCl_2$, 50 mM Tris–HCl buffer adjusted to pH 7.8 at 37 °C, and NaCl as indicated. The reaction components were mixed thoroughly with a Vortex mixer after addition of the enzyme. Reactions proceeded for 15 min at 37 °C and were terminated by placing the reaction tubes in a boiling waterbath for 2 min. The tubes were then transferred to an ice bath, or stored at -80 °C until a 5 μl aliquot of each reaction was chromatographed.

Nucleotides in terminated reaction mixtures were isolated on thin-layer plates of polyethyleneimine cellulose adsorbant. This material proved to be superior to DEAE-cellulose for the separation of nucleotides in mixtures containing appreciable salts. The characteristics of different commercial lots of polyethyleneimine cellulose varied, which necessitated the determination of optimum thickness of the adsorbant layer and the optimum concentration of formic acid solvent for nucleotide resolution for each new lot. Thin-layer plates were eluted with 40% formic acid and then dried before application of samples. After aliquots were applied, the plates were eluted first with water, dried, and then developed with either 10% or 40% formic acid (v/v). This

procedure minimized streaking of nucleotides that occurred during the chromatography of aliquots with high concentrations of salt.

After separation, the nucleotides were located on the polyethyleneimine cellulose by use of a ultraviolet light. It was necessary to add non-radioactive AMP to reaction mixtures in order to locate this nucleotide on the plates. The adsorbant associated with each isolated nucleotide was removed from the thin-layer plate and placed in a scintillation vial to which was added 0.5 ml of 0.3 M HCl. After 30 min, 15 ml of scintillation fluid (60 g naphthalene, 4 g PPO, 0.2 g POPOP, 100 ml methanol, 20 ml ethylene glycol, *p*-dioxane to 1 l) was added and the radioactivity counted in a Packard 3375 scintillation counter. The amount of [^{14}C]ADP converted to [^{14}C]ATP was calculated by dividing the dpm in ATP by the specific activity of [^{14}C]ADP solution and the exchange rate is expressed as μmoles of phosphate exchanged/h per mg protein. Na^+-dependent activity was calculated by substracting the activity in the presence of $MgCl_2$ from the activity in the presence of $MgCl_2$ and NaCl. Determination of the phosphate liberated under the conditions of the exchange reaction indicated that less than 3% of the ATP was hydrolyzed during the assay.

The ^{14}C content of AMP was assayed for each reaction in order to detect adenylate kinase activity. Under the conditions of the standard assay the ^{14}C content of isolated AMP was no greater than that present as an impurity in the [^{14}C]-ADP added to each reaction mixture. When ATP was omitted from the reaction mixtures small quantities of [^{14}C]ATP and [^{14}C]AMP were observed indicating weak adenylate kinase activity. This activity was not stimulated by Na^+ addition and was completely inhibited by 1.0 mM ATP. It was concluded, therefore, that the assays for Na^+-dependent ADP–ATP exchange in the experiments reported here were not complicated by the activity of adenylate kinase in the rat brain microsomes.

RESULTS

In agreement with previous reports[5,6] the NaI-treated rat brain microsomes catalyzed two ADP–ATP exchange reactions; one reaction required only Mg^{2+} while the other reaction proceeded only in the presence of Na^+. In contrast to the report of Stahl[5,6], however, the Na^+-dependent exchange was dependent also on the addition of $MgCl_2$. Optimum Na^+-dependent activity in the absence of oligomycin was observed with 1.0 mM $MgCl_2$, and in the presence of oligomycin 3.0 mM $MgCl_2$.

The relationship between NaCl concentration and the Na^+-dependent exchange activity, determined in two separate experiments, is shown in Fig. 1, A and B.

In both the experiments the Na^+-dependent exchange exhibited a complex response to increasing concentrations of NaCl. An initial maximum response was obtained at 3.0 mM NaCl, and then a series of response plateaus occurred at concentrations ranging up to 200 mM NaCl. The curves in Fig. 1, A and B are essentially the same, except for a small difference in the position of the plateau between 100 and 150 mM NaCl where there are insufficient experimental points to make a precise comparison. In another experiment a similar multiphasic response to increasing NaCl concentration was obtained when Tris buffer was replaced by 0.05 M Tricine at pH 7.5. The addition of oligomycin to reactions in another experiment stimulated the Na^+-dependent exchange activity at all NaCl concentrations tested, but did not abolish the

complex nature of the response to increasing NaCl additions (Fig. 2). These results may be compared with those in Fig. 1 where no oligomycin was included.

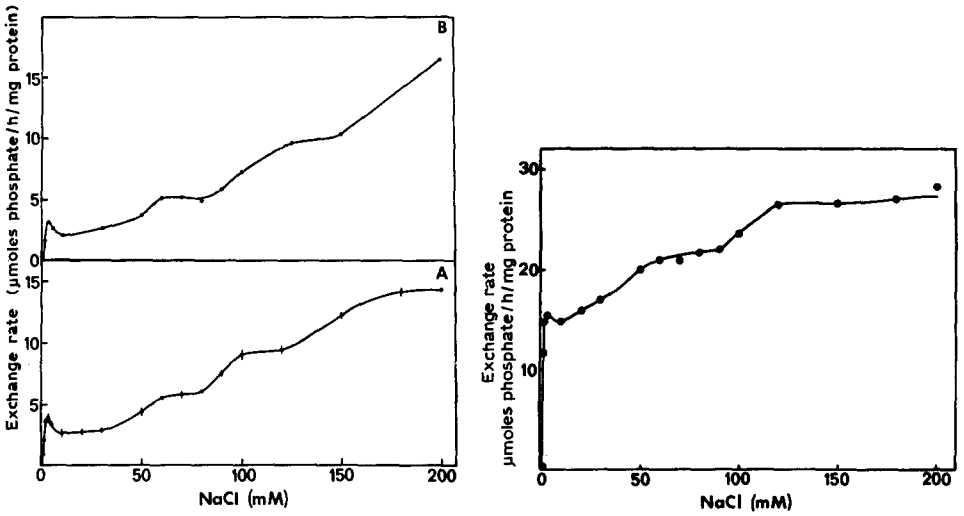

Fig. 1. Effect of increasing NaCl concentrations on the ADP–ATP exchange reaction catalyzed by rat brain microsomes. The standard assay procedure (Materials and Methods) was utilized with with the exception that the NaCl concentration was varied. The experiment in A utilized 4.1 μg microsomal protein per assay and each point represents the mean of three independent determinations of the ADP–ATP exchange reaction. The S.E. of the mean times 2 is shown by a vertical line through each point. The experiment in B utilized 4.1 μg of microsomal protein per assay and each point is the mean of duplicate assays.

Fig. 2. The Na^+ activation of the ADP–ATP exchange reaction in the presence of oligomycin. The assay procedure utilized and the composition of reaction mixtures, with the exception of the addition of 20 μg/ml of oligomycin, are the same as described in Fig. 1. Oligomycin in an ethanolic solution was added to each assay tube and was evaporated to dryness under a stream of N_2 prior to the addition of the other reactants. Each point on the curve represents the mean of triplicate determinations.

The effects of Na^+ on the exchange activity were apparently instantaneous, as the enzyme preparation was not preincubated in the reaction mixture. In an experiment similar to that illustrated in Fig. 2, the enzyme was preincubated in the reaction mixture for 30 min at 0 °C prior to initiating the reaction by the addition of [^{14}C]ADP. The resulting Na^+-response curve was essentially the same as that described in Fig. 2, indicating that preincubation did not alter the Na^+ response in the presence of oligomycin.

In an effort to test the possibility that the complex response to increasing NaCl might have resulted from non-specific effects of ions on the reaction, the univalent cation requirement for the ADP–ATP exchange was determined at selected points on a typical NaCl-response curve. The addition of KCl, RbCl, CsCl, NH_4Cl or LiCl to reaction mixtures utilized for measurement of the exchange reaction failed to substitute for NaCl at 10 or 100 mM in the absence of oligomycin, or at 3 or 200 mM in the presence of oligomycin (Table I). Furthermore, ouabain, a specific inhibitor of ($Na^+ + K^+$)-ATPase and of Na^+ and K^+ transport, inhibited the Na^+-dependent

exchange at 3.0, 100 and 200 mM NaCl (Table II). This is further evidence that $(Na^+ + K^+)$-ATPase, or a component of this enzyme, is responsible for the exchange activity over a broad range of NaCl concentrations utilized in the experiment described in Fig. 1.

TABLE I

CATION SPECIFICITY OF THE ADP–ATP EXCHANGE REACTION AT DIFFERENT LOCATIONS ON THE Na^+ RESPONSE CURVES IN FIGS 1A AND 1B

The standard assay procedure was employed (Materials and Methods) with the exceptions of the additions of oligomycin and salts as indicated.

Salt added	*Rates of exchange (μmoles phosphate/h per mg protein)*			
	Without oligomycin		*With 20 μg/ml oligomycin*	
	With 10 mM salt	*With 100 mM salt*	*With 3 mM salt*	*With 200 mM salt*
None	1.02	1.86	1.41	1.02
NaCl	4.74	8.67	11.71	23.60
KCl	1.15	1.79	1.22	1.08
RbCl	1.25	1.79	1.30	0.73
CsCl	1.36	1.98	1.78	1.49
NH_4Cl	1.21	1.81	1.52	0.80
LiCl	1.15	1.15	1.59	0.47

TABLE II

EFFECT OF Na^+ CONCENTRATION ON THE ADP–ATP EXCHANGE REACTION IN THE PRESENCE AND ABSENCE OF OUABAIN

The standard assay procedure (Materials and Methods) was used with the exceptions that additions of NaCl and ouabain were varied as indicated.

NaCl (mM)	*Rate of exchange (μmoles phosphate/h per mg protein)*	
	Without ouabain	*With 1.0 mM ouabain*
0	1.22	0.88
3	3.00	1.36
100	4.24	1.45
200	6.97	1.32

DISCUSSION

In agreement with previous reports of Stahl[5,6], this research has shown that NaI-treated rat brain microsomal preparations not only possess $(Na^+ + K^+)$-ATPase activity, but also catalyze a Na^+-dependent ADP–ATP exchange reaction. Whereas

the Na^+-dependent exchange reaction investigated by Stahl[5,6] exhibited optimum activity in the absence of added $MgCl_2$, we have observed consistently that the Na^+-dependent exchange activity of a microsomal preparation from a rat brain is completely dependent upon added $MgCl_2$, and is optimal at a concentration of 1.0 mM $MgCl_2$. These results confirm the Mg^{2+} requirement for the exchange reaction that was predicted but not demonstrated by Stahl[6].

The multiphasic response of the ADP–ATP exchange reaction to increasing concentrations of NaCl has not been reported previously. Our unpublished results are in agreement with those of Robinson[17] who showed that the Na^+ saturation curve for the overall $(Na^+ + K^+)$-ATPase reaction by the rat brain microsomes was sigmoidal in nature. No evidence for a multiphasic response to either Na^+ or K^+ by the overall ATPase reaction has been observed. Since the Na^+-dependent ADP–ATP exchange reaction was specific for Na^+ at all concentrations tested (Table I), it is not possible to account for the stimulating effect of NaCl on the basis of increasing ionic strength. The data showing that the exchange reaction was inhibited by ouabain and stimulated by oligomycin over a wide range of NaCl concentrations are consistent with the conclusion that the exchange reaction observed at widely different NaCl concentrations is catalyzed by a single enzyme or enzyme complex.

The possibility was considered that the multiphasic response to increasing NaCl might be associated with a series of Na^+-induced conformational changes in the enzyme complex. Studies with the fluorescent probe, 8-anilino-1-1-naphthalene sulphonic acid indicated that the conformation of the microsomal membrane was altered by the presence of NaCl. No evidence was obtained, however, to indicate that the complex response of the ADP–ATP exchange to increasing NaCl concentration resulted from Na^+-dependent conformational changes in the $(Na^+ + K^+)$-ATPase, or in the microsomes with which the $(Na^+ + K^+)$-ATPase was associated.

The $(Na^+ + K^+)$-ATPase is a multisubunit enzyme system that catalyzes a sequence of interdependent reactions[19]. The multiple binding sites for metal cofactors and for substrates by the $(Na^+ + K^+)$-ATPase may be involved in the allosteric interactions that have been observed by Robinson[17,18] and Tobin *et al.*[20]. In such a complex system kinetic data are difficult to interpret. The possibility exists, however, that the effects of Na^+ on the ADP–ATP exchange reflect interactions between multiple Na^+ binding sites, or Na^+-dependent interactions between enzyme subunits.

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