Biochemical Pharmacology, Vol. 24, pp. 1847 1850. Pergamon Press, 1975. Printed in Great Britain.

STUDIES ON THE MECHANISM OF DOG HEART $(Na^+ + K^+)$-ATPase INHIBITION BY D,L-ASPARTIC ACID AND ITS K^+ AND Mg^{2+} SALTS

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(*Received* 14 *February* 1975; *accepted* 2 *April* 1975)

Abstract—D,L-aspartic acid as well as its K^+ and Mg^{2+} salts have been found to be inhibitory to $Na^+ + K^+$)-ATPase activity of the dog heart microsomal fraction enriched with sarcolemma. According to intensity of the inhibitory effect (noncompetitive) they form the following sequel: mono-K^+-aspartate $\leq$ D,L-aspartic acid $<$ mono-Mg^{2+}-aspartate $<$ a mixture of K^+ and Mg^{2+}-aspartate in a molar ratio of 1:1 (K^+, Mg^{2+}-ASP). The latter mixture is widely used as an agent in cardiac failure. For $(Na^+ + K^+)$-ATPase the salient effects of D,L-aspartic acid and/or its K^+ and Mg^{2+} salts were: (i) decrease in V for ATP as substrate with unchanged K_m; (ii) for Na^+ as an allosteric modifier of $(Na^+ + K^+)$-ATPase activity a decrease in V without any alteration in n as a measure of cooperativity between activating sites; (iii) for K^+ a decrease in V and n as well as an increase in $K_{0.5}$. In the presence of Na^+ and ATP the high affinity of the enzyme for K^+ became reduced by D,L-aspartic acid, lowering at the same time the $K_{0.5}$ value.

Effects like these have also been described for ouabain. The present data show that K^+ and Mg^{2+} salts of D,L-aspartic acid act at a similar locus as does ouabain.

Several compounds and reagents are known to be capable of modifying the Na^+, K^+-dependent ATPase activity in various tissues. So, for example, this activity in the heart is inhibited by cardiac glycosides [1] and stimulated by Co^{2+} ions [2]. Recently, we could also show that D,L-aspartic acid as well as a defined mixture of its K^+ and Mg^{2+} salts, which is widely used as a drug in the failing heart, are specific inhibitors of dog heart $(Na^+ + K^+)$-ATPase and that the inhibition of this enzyme activity by ouabain is lowered in the presence of aspartate [3,4].

The aim of the work presented in the paper was to establish some of the outstanding characteristics of (Na^+, K^+)-ATPase inhibition by D,L-aspartic acid as well as its K^+ and Mg^{2+} salts and to study the mode of interaction between the enzyme molecule, aspartate and ouabain.

MATERIAL AND METHODS

The fraction containing $(Na^+ + K^+)$-stimulated, Mg^{2+}-dependent ATPase was isolated from dog heart left ventricles (of approx 20 g) according to the procedure of Matsui and Schwartz [5].

It was found to be contaminated by less than 3% of succinate dehydrogenase activity of the mitochondrial fraction [6].

To determine ATPase activity 70 μg protein was incubated in a shaking water bath of 37° for 10 min in a total volume of 1 ml containing 50 mM Tris–HCl, pH 7·4, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl and 20 mM KCl. Reaction was started by adding 0·1 ml of 40 mM Tris–ATP and stopped by 1 ml of 12% trichloracetic acid. The amount of P_i released into the medium through ATP hydrolysis was estimated in the protein-free supernatant by the method of Tausski and Shorr [7]. At each measurement, correction was made for spontaneous hydrolysis of ATP. Protein concentration was measured according to Lowry *et al.* [8]. Total ATPase activity refers to the activity measured in the presence of Mg^{2+}, Na^+ and K^+ ions. Mg^{2+}-dependent ATPase activity is that obtained in the absence of Na^+ and K^+ ions. $(Na^+ + K^+)$-ATPase activity is calculated by subtracting Mg^{2+}-dependent ATPase from total activity [9].

Experimental points are the average of six or more experiments performed in triplicate. Values from the Hill plots of n and $K_{0.5}$ were calculated from the equations for straight lines obtained by the method of least squares with standard deviations calculated as described by Robinson [9].

The stock solution of D,L-aspartic acid and/or K^+ and Mg^{2+}-aspartate in a mutual ratio of 1 : 1 contained 4·2 m-moles of the substance(s) in 10 ml; the pH was adjusted with Tris–HCl to 7·4. All reagents used were of analytical grade, purchased from Sigma Ltd. and Merck. The solutions were made in water that had been redistilled from an all-glass still.

RESULTS

Effect of D,L-*aspartic acid or its* K^+ *and/or* Mg^{2+} *salts on* Mg^{2+}*-dependent,* $(Na^+ + K^+)$*-activated ATPase.* This effect was assayed taking into account the fact that in all the applied concentrations the above substance appeared to have no inhibitory effect on the Mg^{2+}-activated enzyme [4]. Table 1 shows

Table 1. Effect of D,L-aspartic acid and its K^+ and Mg^{2+} salts on Mg^{2+}-dependent, Na^+, K^+-activated ATPase in the dog heart

Substance	Concentration (M)	Inhibition of ($Na^+ + K^+$)-ATPase activity (% of control value)
D,L-aspartic acid	$1{\cdot}0 \times 10^{-3}$	6·2
	$5{\cdot}0 \times 10^{-3}$	20·0
	$1{\cdot}0 \times 10^{-2}$	36·1
	$5{\cdot}0 \times 10^{-2}$	52·2
K^+-and Mg^{2+}-aspartate*	$4{\cdot}2 \times 10^{-4}$	7·5
	$8{\cdot}4 \times 10^{-4}$	19·5
	$1{\cdot}3 \times 10^{-3}$	38·6
	$1{\cdot}7 \times 10^{-3}$	54·2
	$2{\cdot}1 \times 10^{-3}$	60·1
Mg^{2+}-aspartate	$1{\cdot}4 \times 10^{-4}$	1·5
	$5{\cdot}0 \times 10^{-4}$	7·7
	$1{\cdot}0 \times 10^{-3}$	34·6
	$5{\cdot}0 \times 10^{-3}$	53·9
	$1{\cdot}0 \times 10^{-2}$	65·4
K^+-aspartate	$2{\cdot}8 \times 10^{-4}$	—
	$5{\cdot}0 \times 10^{-4}$	3·9
	$1{\cdot}0 \times 10^{-3}$	7·7
	$5{\cdot}0 \times 10^{-3}$	16·9
	$1{\cdot}0 \times 10^{-2}$	26·2

The standard assay conditions given in Methods were used, expressing the enzyme activity in μmoles P_i liberated per mg protein per hour. Each result represents the average of 10 estimations.

* A mixture consisting of mono-K^+-D,L-aspartate and mono-Mg^{2+}-D,L-aspartate in a molar ratio of 1 : 1, i.e. in a ratio which is present in the commercial preparation Tromcardin® (Trommsdorf, Aachen, German Federal Republic).

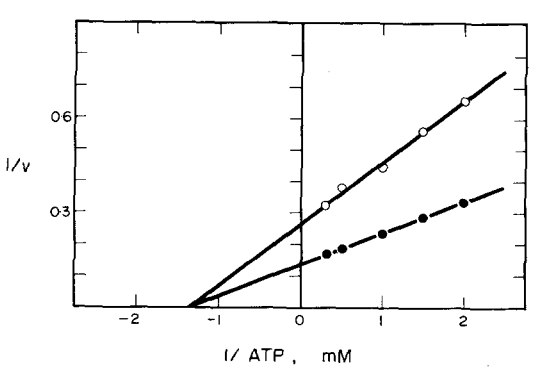

Fig. 1. Inhibition of heart ($Na^+ + K^+$)-ATPase by K^+- and Mg^{2+}-salts of D,L-aspartic acid. The rate of P_i production in μmoles per mg protein per hour is plotted against ATP concentration in mM in the Lineweaver–Burk form. Inhibitor was applied in a concentration of 1·68 mM and represented a mixture of mono-K^+-D,L-aspartate and mono-Mg^{2+}-D,L-aspartate in a molar ratio of 1:1. The Mg^{2+} ATP ratio was constantly maintained taking 1·25 at all ATP concentrations which varied from 0·5 to 3 mM. The other assay conditions are given in Methods. Each point represents the average of six estimations; lines were drawn by the method of least squares. ●—Controls, ○—K^+- and Mg^{2+}-aspartate.

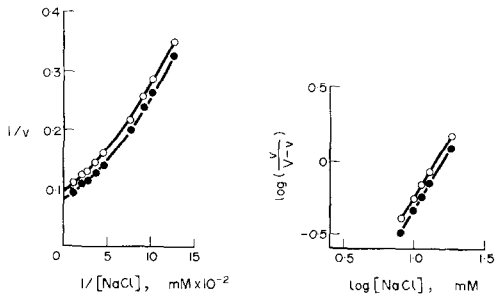

Fig. 2. Na^+ activation of the Na^+, K^+-dependent ATPase. The enzyme preparation was incubated at 37° under standard conditions given in Methods but with concentrations of NaCl shown (●). Parallel experiments were performed in the presence of 5·0 mM D,L-aspartic acid (○). Data are presented in the left hand panel in the form of a Lineweaver–Burk plot and in the right hand panel in the form of a Hill plot, with the straight lines drawn by the method of least squares. Each point represents an average of six or more estimations.

that D,L-aspartic acid and its K^+-salts are less inhibitory to ($Na^+ + K^+$)-ATPase activity than its Mg^{2+} or its K^+ and Mg^{2+} salts together [3, 4]. The corresponding concentrations for half maximal inhibition and K_i values were established graphically. They amounted to

$$I_{50\ asp.ac.} = 45\ mM$$

and

$$I_{50,K^+\text{-and } Mg^{2+}\text{-asp.}} = 1{\cdot}68\ mM.$$

On the other hand, a similar K_i of 0·56 mM has been found for both aspartic acid and the mixture of its K^+ and Mg^{2+} salts respectively. Using glutamic acid instead of aspartic acid in a concentration range of 10^{-4} M–10^{-2} M no inhibition of ($Na^+ + K^+$)-ATPase could be observed.

Inhibition of enzyme activity by K^+ and Mg^{2+} salts of aspartic acid proved to be noncompetitive, differing only in V_i and exhibiting Michaelis–Menten kinetics (Fig. 1) [3, 4].

In view of what has been said above all the following experiments, with the exception of studies of the

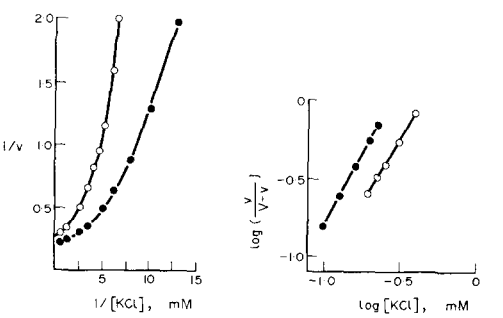

Fig. 3. K^+ activation of the Na^+,K^+-dependent ATPase. The enzyme preparation was incubated at 37° in the standard medium (see Methods) but with the concentrations of KCl shown (●). Parallel experiments were performed in the presence of 5·0 mM D,L-aspartic acid (○). Data are presented as in Fig. 2.

Table 2. Kinetic Parameters of Na^+- and K^+-activation of Na^+, K^+-dependent ATPase

Conditions	Additions	Kinetic parameters V_{max}	$K_{0.5}$ (mM)	n
For Na^+	None	12·00 ± 0·92	5·90 ± 0·63	1·57 ± 0·04
With 20 mM KCl	5·0 mM D,L-aspartic acid	10·00 ± 0·80	6·07 ± 0·67	1·55 ± 0·04
For K^+	None	5·55 ± 0·50	0·48 ± 0·04	1·88 ± 0·05
With 100 mM NaCl	5·0 mM D,L-aspartic acid	4·16 ± 0·52	0·66 ± 0·07	1·66 ± 0·04

Experiments were performed as in Figs. 2 and 3 either with a constant concentration of KCl (20 mM) or with a constant concentration of NaCl (100 mM). Data are presented ± S.D.

effects of D,L-aspartic acid on the activating action of Na^+ and K^+ ions, have been performed with the more inhibitory mixture of K^+ and Mg^{2+} salts. The error brought about by changing in this way the optimal K^+ and Mg^{2+} concentrations present in the assay system proved to be negligible [4].

Influence of D,L-*aspartic acid on allosteric properties of* $(Na^+ + K^+)$-ATPase. In studying the activation of $(Na^+ + K^+)$-ATPase by Na^+ and K^+ in the presence and/or absence of D,L-aspartic acid at constant optimal NaCl (100 mM for K^+-activation) and KCl (20 mM for Na^+-activation) concentrations—where competitive interactions between Na^+ and K^+ might be expected to be minimized [10, 11]—sigmoidal Lineweaver–Burk plots concave upward and corresponding Hill plots, in which the slope (n) is greater than 1, have been obtained (Figs. 2 and 3).

In Hill's equation, n has been interpreted as a measure of both the number of sites for the reactant and the intensity of interaction between them; it is generally used as a criterion of cooperativity. Our results (Table 2) demonstrate that, similarly as it has been reported for ouabain, D,L-aspartic acid will alter the cooperative homotropic effect of K^+ by decreasing n and also its heterotopic effect by decreasing V_{max} [12–16].

When studying the interaction between the inhibitor, Na^+ ions and the enzyme, a decrease in V_{max} by 16·6 per cent and by a 3 per cent increase of Na^+ concentration to half maximal activation ($K_{0.5}$) could be noted.

Interaction between K^+ *and* Mg^{2+} *aspartate and ouabain on* $(Na^+ + K^+)$-*ATPase.* In order to check whether both ouabain and K^+ and Mg^{2+} aspartate are actually acting on similar, or different, binding sites the sum of individual inhibitions with both inhibitors $i_1 + i_2$ was compared with the inhibition produced by the inhibitors acting together $i_{1,2}$, using the formulae according to Webb [17].

$$i_1 + i_2 = \frac{[I_1]' + [I_2]' + 2\{[I_1]' \times [I_2]'\}}{1 + [I_1]' + [I_2]' + \{[I_1]' \times [I_2]'\}} \quad \text{(a)}$$

$$i_{1,2} = \frac{[I_1]' + [I_2]'}{1 + [I_1]' + [I_2]'} \quad \text{(b)}$$

$$\alpha = \frac{[I_1]' \times [I_2]'}{(x - y) \times \{[I_1]' - [I_2]'\}} \quad \text{(c)}$$

where

$$[I_1]' = \frac{I_1}{K_{i_1}}; \qquad [I_2]' = \frac{I_2}{K_{i_2}};$$

and $x = y = 1$ for 50% inhibition and when both inhibitors are noncompetitive.

The results obtained are shown in Table 3. In the case when both inhibitors were present in I_{50} concentrations (established graphically) $i_1 + i_2$ [equation (a)] exceed $i_{1,2}$ [equation (b)], i.e. the criterion stated for the binding of inhibitors at the same site has been fulfilled. In fact, using the criteria stated by Webb [17] for the above-quoted case, when both inhibitors are non-competitive, the interaction constant [equation (c)] giving the effect of binding of one substance on the binding of another at the active site is infinite ($\alpha = \infty$).

DISCUSSION

In our earlier studies [3, 4] it was demonstrated that inhibition by ouabain of $(Na^+ + K^+)$-ATPase was considerably decreased in the presence of D,L-aspartic acid or its K^+ and Mg^{2+} salts. Further we could show that the latter substances are inhibitory by themselves to $(Na^+ + K^+)$-ATPase activity. As it appears from the present results, according to intensity of their inhibitory action their sequence is mono-K^+-aspartate ⩽ D,L-aspartic acid < mono-Mg^{2+}-aspartate < a mixture of K^+ and Mg^{2+}-aspartate in a molar ratio of 1 : 1 (K^+, Mg^{2+}-ASP). However, K^+ and Mg^{2+} ions proved to be without any influence on $(Na^+ + K^+)$-ATPase activity even in concentrations at which, if they are components of K^+- and Mg^{2+}-aspartate, these substances have been found to be inhibitive. Thus, the former sequence seems to follow the rule, the less the complex is dissociated in aqueous solutions the more it is inhibitory. Nevertheless, in the case of a mixture of K^+ and Mg^{2+} salts some synergism is likely to be operative.

It appears from kinetics studies that similarly as it could be expected for ouabain, K^+ and Mg^{2+} salts

Table 3. Interaction between K^+-and Mg^{2+}-aspartate and ouabain on $(Na^+ + K^+)$-ATPase

I_1 $K^+ + Mg^{2+}$-aspartate (mM)	I_2 Ouabain (mM)	$i_1 + i_2$	$i_{1,2}$
0·84	0·10	2·68	0·98
1·68	0·10	1·51	0·98
0·84	0·004	1·35	0·82
1·68*	0·004*	1·50*	0·86*
0·84	0·001	1·03	0·69
1·68	0·001	1·18	0·79

* Results when both $[I_1]$ and $[I_2] = [I_{50}]$.

of aspartic acid do not compete either with ATP as substrate or with Na^+ and K^+, and that the aspartic moiety will influence the cooperative homotropic and heterotopic effects on the enzyme of K^+ [9, 16]. Moreover, it could be demonstrated in the present study that both ouabain and D,L-aspartic acid or its K^+ and Mg^{2+} salts are acting at similar sites on the enzyme molecule.

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