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Effects of Derivatives of Hydroxypyruvaldehyde Phenylosazone on the Ca^{2+}-Adenosine Triphosphatase Activity of Porcine Erythrocyte Membrane

TOSHIKATSU NAKABAYASHI,*,[a] HIROH IKEZAWA,[a] HARUKI OGAWA,[b] MINORU SHINDO,[b] and ISAO MATSUNAGA[b]

Faculty of Pharmaceutical Sciences, Nagoya City University,[a] Mizuho-ku, Nagoya, 467, Japan and New Drug Research Laboratories, Chugai Pharmaceutical Co., Ltd.,[b] Toshima-ku, Takada, Tokyo 171, Japan

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Thirteen derivatives of hydroxypyruvaldehyde phenylosazone were tested for their effects on the Ca^{2+}-adenosine triphosphatase (ATPase) activity of porcine erythrocyte membranes. Seven drugs including hydroxypyruvaldehyde phenylosazone itself and the *m*-CH_3, *p*-CH_3, *o*-Cl, *p*-Cl, 3,4-di-Cl and *p*-Br derivatives, inhibited Ca^{2+}-ATPase activity, while other derivatives such as *o*-CH_3, 2,4-di-Cl, 2,5-di-Cl, *o*-NO_2, *m*-NO_2 and *p*-NO_2 derivatives were essentially without effect. Hydroxypyruvaldehyde *p*-chlorophenylosazone was the most potent inhibitor, inhibiting the enzyme activity completely at 1 mM, whereas carbonylcyanide *m*-chlorophenylhydrazone, a typical inhibitor of Ca^{2+}-ATPase, inhibited the activity by 85% at the same concentration.

Keywords——hydroxypyruvaldehyde phenylosazone; porcine erythrocyte membrane; Ca^{2+}-ATPase inhibitor; erythrocyte Ca^{2+}-ATPase

Ca^{2+}-Adenosine triphosphatase (ATPase) present in the plasma membrane of erythrocytes possesses a Ca^{2+} transport activity, the so-called Ca^{2+} pump, which keeps the intracellular Ca^{2+} concentration below 1 μM.[1] An activator of this enzyme was first reported in the crude hemolysates by Bond and Clough,[2] and was identified as calmodulin, which regulates the activity of Ca^{2+}-ATPase.[3]

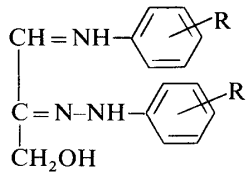
Recently, Hayashi *et al.*[4] showed that incubation of erythrocyte ghosts with carbonylcyanide *m*-chlorophenylhydrazone (CCCP) in the presence of Ca^{2+} resulted in the inactivation of Ca^{2+}-ATPase activity. On the other hand, we reported the effects of derivatives of hydroxypyruvaldehyde phenylosazone on the osmotic fragility and morphology of bovine erythrocyte membrane.[5] Since CCCP is structurally related to the hydroxypyruvaldehyde phenylosazones, we examined the effects of these compounds on the Ca^{2+}-ATPase activity of porcine erythrocyte membrane.

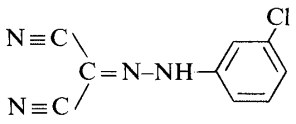
Experimental

Drugs and Other Chemicals——Hydroxypyruvaldehyde phenylosazone (**1**) and its derivatives (**2**—**13**) shown in Table I were prepared according to the method described in a previous report.[5] Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was purchased from Calbiochem, U.S.A. All other reagents used were of analytical grade.

Preparation of Porcine Erythrocyte Membranes——Fresh, defibrinated porcine blood was centrifuged at $2000 \times g$ for 10 min and the plasma and buffy coat were removed by aspiration. The collected erythrocytes were washed three times with phosphate-buffer saline (pH 7.4) which was made isotonic by addition of 150 mM NaCl to 10 mM Na-phosphate buffer. Then the erythrocyte membranes were prepared by the method of Dodge *et al.*[6] as modified by Hayashi and Penniston.[7] The erythrocyte membranes obtained were suspended in 20 mM imidazole–HCl buffer (pH 7.4) at the concentration of 5 mg protein/ml, frozen quickly in liquid N_2, and stored at -20 °C.

TABLE I. Structural Formulae of Hydroxypyruvaldehyde Phenylosazones and Carbonylcyanide *m*-Chlorophenylhydrazone





carbonylcyanide *m*-chlorophenylhydrazone[a]

Compd. No.	R	Solvent	Compd. No.	R	Solvent
1	H	Methanol	**8**	2,5-di-Cl	Methanol
2	*o*-CH_3	Methanol	**9**	3,4-di-Cl	Methanol
3	*m*-CH_3	Methanol	**10**	*p*-Br	Methanol
4	*p*-CH_3	Methanol	**11**	*o*-NO_2	DMSO
5	*o*-Cl	Methanol	**12**	*m*-NO_2	DMSO
6	*p*-Cl	Methanol	**13**	*p*-NO_2	DMSO
7	2,4-di-Cl	Methanol			

a) This compound was dissolved in dimethylsulfoxide (DMSO).

Ca^{2+}-ATPase Assay——ATPase activity in erythrocyte membranes shows an absolute requirement for Mg^{2+}. Thus the basal activity was obtained in the presence of Mg^{2+} (Mg^{2+}-ATPase) and stimulated by Ca^{2+} (Ca^{2+}-ATPase) or by $Na^+ + K^+$ (Na^+, K^+-ATPase). Ca^{2+}-ATPase activity was estimated by subtracting the ATPase activity in the presence of Mg^{2+} from that measured in the presence of both Ca^{2+} and Mg^{2+}. According to the modified method of Jarrett and Penniston,[8] Ca^{2+}-ATPase activity was measured as follows. The reaction mixture (0.5 ml) contained 6 mM $MgCl_2$, 0.8 mM $CaCl_2$, 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 6 mM ATP and the enzyme (250 μg) in 30 mM imidazole–HCl buffer (pH 7.0). In some cases, 100 mM NaCl, 20 mM KCl and 0.1 mM ouabain were added to the reaction mixture. After incubation at 37 °C for 5 min, the enzyme reaction was started by the addition of adenosine triphosphate (ATP). Then, the enzyme reaction was terminated at 30 min by the addition of 0.7 ml of a solution containing 0.5 M H_2SO_4, 0.5% (w/v) ammonium molybdate and 2% (w/v) sodium dodecyl sulfate with 20 μl of a solution containing sodium metabisulfite, 1.2% (w/v) sodium sulfite and 0.2% (w/v) 1-amino-2-naphthol-4-sulfonic acid. The reaction mixtures were allowed to stand at room temperature for 20 min, then the amounts of inorganic phosphate liberated from ATP were measured spectrophotometrically at 650 nm. The drugs (**1**—**13**), *i.e.*, hydroxypyruvaldehyde phenylosazone and its derivatives, and CCCP, were dissolved in methanol or dimethylsulfoxide, and added to the reaction mixture to determine the effects on the activity of Ca^{2+}-ATPase.

Assay of Protein——Protein was determined by the method of Lowry *et al.*[9] with bovine serum albumin as a standard.

Results and Discussion

Effects of Hydroxypyruvaldehyde Phenylosazones and Carbonylcyanide *m*-Chlorophenylhydrazone on the Determination of Inorganic Phosphate

Prior to analysis of the effects of drugs on Ca^{2+}-ATPase activity, the influence of the drugs, CCCP and solvents such as methanol and dimethylsulfoxide (DMSO) was examined on the determination of inorganic phosphate liberated from ATP by the action of Ca^{2+}-ATPase. At first, we employed the method of Eibl and Lands[10] with a slight modification[11] to determine the concentration of liberated inorganic phosphate, but this method was unsuccessful because of interference by the porcine erythrocyte membrane preparation. Thus, we adopted the method of Fiske and Subbarow,[12] a typical method for Pi determination, as modified by Jarrett and Penniston.[8] All the compounds tested, *i.e.*, drugs (**1**—**13**), CCCP, solvent and erythrocyte membranes, were essentially without effect on the determination of inorganic phosphate in the range of 50 to 500 nmol.

Effects of Methanol and Dimethylsulfoxide on Ca^{2+}-ATPase Activity

The effects of methanol and DMSO, the solvents for drugs and CCCP, on the activity of Ca^{2+}-ATPase of porcine erythrocyte membrane were examined. The reaction mixtures containing various amounts of methanol or DMSO were incubated at 37 °C for 30 min. The addition of methanol or DMSO at the concentration of 10% (v/v) reduced the Ca^{2+}-ATPase activity to 60% of the control, but significant inhibition was not observed when the concentration of methanol or DMSO was below 5% (v/v). Thus we used these solvents at 5% (v/v) in the Ca^{2+}-ATPase assay system.

Effects of Derivatives of Hydroxypyruvaldehyde Phenylosazone and Carbonylcyanide *m*-Chlorophenylhydrazone on Ca^{2+}-ATPase Activity

Table II shows the effects of drugs on the Ca^{2+}-ATPase activity of porcine erythrocyte membrane. Among the drugs tested, 1 mM hydroxypyruvaldehyde phenylosazone (**1**) and its *p*-Br derivative (**10**) inhibited Ca^{2+}-ATPase activity by 93 and 60%, respectively. Moreover, the *p*-Cl derivative (**6**) completely abolished the enzyme activity at 1 mM, whereas the *o*-Cl derivative (**5**) inhibited the activity by 83% at the same concentration. Among the dichloro derivatives (**7**—**9**), which were less soluble than the monochloro derivatives, only hydroxypyruvaldehyde 3,4-dichlorophenylosazone (**9**) inhibited the enzyme activity, while 2,4- and 2,5-dichloro derivatives (**7**, **8**) were without effect. The addition of hydroxypyruvaldehyde *o*-methylphenylosazone (**2**) did not affect the enzyme activity, whereas both *m*-CH_3 (**3**) and *p*-CH_3 (**4**) derivatives were inhibitory. Other drugs, such as NO_2-derivatives (**11**—**13**), were essentially without effect. According to Hayashi *et al.*,[4] CCCP is a potent inhibitor for Ca^{2+}-

TABLE II. Effects of the Drugs on the Ca^{2+}-ATPase Activity of Porcine Erythrocyte Membranes

Compd. No.	Final concentration	ATPase activity (nmol/mg/min)[a]					Relative activity[b] (%)
		Mg^{2+}	Mg^{2+}-Ca^{2+}	Mg^{2+}-Na^+-K^+ (−ouabain)	Mg^{2+}-Na^+-K^+-Ca^{2+} (−ouabain)	Mg^{2+}-Na^+-K^+-Ca^{2+} (+ouabain)	
Control (methanol)		6.8	25.1	10.8	40.2	37.5	100
1	1	7.0	8.2	9.3	10.2	9.6	7
2	1	7.0	25.0	11.2	41.2	38.5	98
3	1	6.7	10.0	9.0	14.3	11.7	18
4	1	7.1	11.5	10.7	18.1	16.2	24
5	1	6.9	10.0	9.1	15.2	13.6	17
6	1	6.7	6.7	7.3	8.8	8.7	0
7	0.1	6.9	25.1	11.3	39.6	35.1	99
8	0.1	6.9	24.8	10.8	40.7	38.0	98
9	0.1	6.8	19.8	10.4	34.3	30.6	71
10	1	7.0	14.3	10.7	23.6	20.5	40
Control (DMSO)		6.4	24.6	10.0	38.6	35.2	100
11	1	6.5	24.5	10.2	39.0	36.1	99
12	1	6.7	23.9	10.3	37.4	34.4	95
13	1	6.3	24.1	10.1	38.1	35.8	98
Carbonylcyanide *m*-chlorophenylhydrazone	1	6.0	8.8	9.4	13.8	11.6	15

a) The reaction mixture (0.5 ml) for Ca^{2+}-ATPase contained 6 mM $MgCl_2$, 0.8 mM $CaCl_2$, 0.5 mM EGTA, 6 mM ATP, the enzyme (250 μg) and the drug in 30 mM imidazole-HCl buffer (pH 7.0). In order to determine other ATPase activities, 100 mM NaCl, 20 mM KCl and 0.1 mM ouabain were added to the reaction mixture. After incubation at 37 °C for 5 min, the enzyme reaction was started by the addition of ATP. Then, the enzyme activity was determined as described in the text. *b*) Relative activity of Ca^{2+}-ATPase was calculated as follows:

$$\frac{(Mg^{2+}\text{-}Ca^{2+}\text{-ATPase activity} - Mg^{2+}\text{-ATPase activity}) \text{ in the presence of drug}}{(Mg^{2+}\text{-}Ca^{2+}\text{-ATPase activity} - Mg^{2+}\text{-ATPase activity}) \text{ in control}} \times 100.$$

TABLE III. The Concentration of Drugs Required for 50% Inhibition (ID_{50}) of Ca^{2+}-ATPase Activity

Compd. No.	ID_{50} (mM)	Compd. No.	ID_{50} (mM)
1	0.44	**6**	0.10
3	0.60	**10**	0.68
4	0.49	Carbonylcyanide *m*-chlorophenylhydrazone	0.40
5	0.40		

The systems for Ca^{2+}-ATPase activity assay and calculation of relative activity were the same as described in Table II. The concentration of drugs in the assay mixture was varied from 0.1 to 1 mM.

ATPase of porcine erythrocyte membranes, inhibiting the enzyme activity completely at 1 mM. In our experiments, CCCP at 1 mM inhibited Ca^{2+}-ATPase activity by 85%.

Table III shows the concentration of drugs required for 50% inhibition of Ca^{2+}-ATPase, *i.e.*, ID_{50}. Hydroxypyruvaldehyde *p*-chlorophenylosazone (**6**) was the most potent inhibitor. The data indicate that these drugs are available for studies on the function of Ca^{2+}-ATPase of erythrocyte membranes.

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References

1) V. Niggli, E. S. Adunyah, J. T. Penniston and E. Carafoli, *J. Biol. Chem.*, **256**, 395 (1981); V. Niggli, E. S. Adunyah and E. Carafoli, *ibid.*, **256**, 8588 (1981).
2) G. H. Bond and D. L. Clough, *Biochim. Biophys. Acta*, **323**, 592 (1973).
3) R. M. Gopinath and F. F. Vincenzi, *Biochem. Biophys. Res. Commun.*, **77**, 1203 (1977); H. W. Jarrett and J. T. Penniston, *ibid.*, **77**, 1210 (1977).
4) H. Hayashi, G. A. Plishker and J. T. Penniston, *Biochim. Biophys. Acta*, **394**, 145 (1975).
5) H. Ikezawa, M. Mizuno, T. Nakabayashi, H. Ogawa, M. Shindo and I. Matsunaga, *Chem. Pharm. Bull.*, **30**, 959 (1982).
6) J. T. Dodge, C. Mitchell and D. J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).
7) H. Hayashi and J. T. Penniston, *Arch. Biochem. Biophys.*, **159**, 563 (1973).
8) H. W. Jarrett and J. T. Penniston, *J. Biol. Chem.*, **253**, 4676 (1978).
9) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 256 (1951).
10) E. Eibl and W. E. M. Lands, *Anal. Biochem.*, **30**, 51 (1969).
11) T. Nakabayashi and H. Ikezawa, *J. Biochem.* (Tokyo), **84**, 351 (1978).
12) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).