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Ca^{2+} REGULATED MODULATOR PROTEIN INTERACTING AGENTS: INHIBITION OF Ca^{2+}-Mg^{2+}-ATPase of HUMAN ERYTHROCYTE GHOST

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SUMMARY

Agents such as N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), and its derivatives, chlorpromazine and amitriptyline that interact with calcium-regulated modulator protein were found to inhibit not only Ca^{2+} dependent cyclic nucleotide phosphodiesterase but also Ca^{2+}-Mg^{2+}-ATPase of human erythrocyte ghosts. I_{50} values of modulator interacting agents for testis modulator-activated, brain modulator-activated and erythrocyte modulator-activated-ATPase are indistinguishable. However, I_{50} of W-7 for troponin C-activated-ATPase is lower than that for modulator-activated ATPase. The specificity of these agents toward modulator-related enzyme reaction is also shown by the negative effect on modulator-unrelated enzyme system such as erythrocyte ghost protein kinase and Mg^{2+}-ATPase. These agents provide a useful tool for elucidating the physiological role of modulator.

INTRODUCTION

Kakiuchi et al. (1) and Cheung (2) independently demonstrated an activator protein (Ca^{2+}-regulated modulator protein, modulator) for cyclic nucleotide phosphodiesterase in rat brain. Both the enzyme activation by modulator and the association between the enzyme and modulator depend on the Ca^{2+} in the reaction media (3).

In 1973, Bond and Clough described the existence of a soluble protein activator of erythrocyte Ca^{2+}-Mg^{2+}-ATPase (4). The presence of an ATPase activator was confirmed by Luthra et al. (5, 6) who further showed the protein to be acidic and heat stable.

Recently several investigators have suggested that the ATPase activator resembles or is identical to the modulator of cyclic nucleotide phosphodiesterase (7, 8). Both ATPase activator and modulator have biological

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cross-reactivity, similar ultraviolet absorption spectra, isoelectric points, similar of identical molecular weights and very similar amino acid composition including the existence of unusual amino acid, trimethyllysine (9).

N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and several drugs such as chlorpromazine, chlorprothixene, amitriptyline and desipramine were reported to be selective inhibitors of modulator-induced actomyosin superprecipitation and stimulation of phosphodiesterase (10, 11, 12, 13, 14).

In the present paper, we present evidence that these several compounds also inhibit erythrocyte Ca^{2+}-Mg^{2+}-ATPase via interfering with their activation by modulator.

MATERIALS AND METHODS

Sephadex G-100 was purchased from Pharmacia Fine Chemicals. ATP (2Na), histone type II, bovine serum albumin fraction V and EGTA were obtained from Sigma Co. DEAE-cellulose (DE-52) was from Whatman Co. [$r^{32}P$]-ATP (specific activity : 19.8 Ci/mmol) was from Radiochemical Center, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and its derivatives (W-8, W-9, W-10) were kindly provided by Banyu Pharmaceutical Co., chlorpromazine by Shionogi Pharmaceutical Co., and amitriptyline by Merk Sharp & Dohme. The concentration of a drug which produced 50% inhibition of Ca^{2+}-Mg^{2+}-ATPase activation in the presence of 3 μg/tube modulator or 15 μg/tube troponin C is defined as I_{50} for that drug. I_{50} (basal Ca^{2+}-Mg^{2+}-ATPase) is defined as the concentration of drug which produced 50% inhibition of ATPase in the absence of modulator.

Preparation of hemolysate and membranes: Outdated human blood was obtained from the local blood bank. Erythrocyte membranes were prepared according to Luthra et al. (5). Briefly, packed erythrocytes were washed three times with 0.172 M Tris-HCl, pH 7.6. The buffy coat was carefully removed. Erythrocytes hemolysis was induced by addition of 280 ml of distilled water to 20 ml of a 50% suspension of erythrocytes in 0.172 M Tris-HCl, pH 7.6. After centrifugation at 27,000 x g for 30 min, supernatant (hemolysate) was stored at -20°C. The pellet was washed 3 times with 20 mM Tris-HCl buffer, pH 7.6 and resultant almost white ghosts were washed once with 100 ml of 18 mM-18 mM histidine-imidazole buffer, pH 7.1.

Ca^{2+}-Mg^{2+}-ATPase assay: The ATPase assay used here was essentially the same as that described by Gopinath and Vincenzi (8). Reaction mixture in a final incubation volume of 1.0 ml contained 2 mM ATP, 18 mM-18 mM histidine-imidazole buffer, pH 7.1, 0.1 mM EGTA, 3 mM $MgCl_2$, 80 mM NaCl, 15 mM KCl, 0.1 mM ouabain (Sigma. Co.) and 110 μg of erythrocyte ghost proteins. $CaCl_2$ (0.2 mM) was present in all tubes except in those for the determination of Mg^{2+}-ATPase. The reaction was started by addition of ATP and carried out at 37°C for 30 min. Inorganic phosphate liberated was determined using the method of Fiske and Subbarow (15).

Protein concentration was determined by the method of Lowry et al. (16). Bovine serum albumin was used as a standard.

Preparation of modulator and troponin C: Porcine brain and rat testis modulator were prepared as described previously (17). Troponin was prepared from rabbit skeletal muscle as described Ebashi et al. (18) and troponin C was isolated by chromatography on DEAE-cellulose according to the method described by Perry and Cole (19).

Protein phosphorylation: The incorporation of ^{32}P into proteins from [$r^{32}P$]-ATP was determined by a modification of the procedure described by Hosey and Tao (20). In addition to erythrocyte membranes, the incubation mixture contained the following in a final volume of 0.25 ml; 18 mM-18 mM imidazole-histidine buffer, pH 7.1: 0.1 mM EGTA; 0.2 mM [$r^{32}P$]-ATP; ± 2 μM cyclic AMP; ± testis modulator (3 μg/tube); and ± exogenous protein substrate. The study of autophosphorylation was conducted at substrate amounts (250 μg) of membrane proteins. When incorporation of ^{32}P into exogenous substrate was to be determined the incubation tube contained 250 μg histone and catalytic amounts (25 μg) of membrane proteins.

Hemolysis procedure: Fresh human erythrocytes were washed three times with normal saline as described above. 1.7×10^9 washed erythrocytes were suspended in 1 ml of normal saline containing varying doses of selective inhibitors and incubated at 37°C for 60 min. The reaction was terminated by the addition of 1.5 ml of ice cold normal saline. Then, reaction mixture was centrifuged at 2,000 rpm for 15 min. Degree of hemolysis was estimated by measurement of absorbance at 540 nm of resultant supernatant.

RESULTS

Effect of modulator interacting agents on erythrocyte Ca^{2+}-Mg^{2+}-ATPase activity

In agreement with Gopinath and Vincenzi (8), both modulator (3.0 μg) and RBC cytoplasmic activator (i.e., dialyzed, boiled hemolysate, 330 μg) increased Ca^{2+}-Mg^{2+}-ATPase activity by about 150% over control. Rabbit skeletal muscle troponin C (15 μg) caused an increase of 65% over control. The activations are selective because these proteins had no significant effect on Mg^{2+}-ATPase as reported previously (7, 8, 9). Effects of W-7, W-8, W-9, W-10, chlorpromazine and amitriptyline on the Ca^{2+}-Mg^{2+}-ATPase activity were examined in the presence or absence of Ca^{2+} and modulator. A typical experiment was shown in Fig. 1. These agents produced concentration-dependent inhibition of Ca^{2+}-Mg^{2+}-ATPase activation by modulator but failed to inhibit Mg^{2+}-(Ca^{2+} independent)-ATPase. The concentrations (I_{50}) of these agents producing 50% inhibition of the ATPase activation by Ca^{2+}-modulator, basal Ca^{2+}-Mg^{2+}-ATP (i.e. without modulator) and Mg^{2+}-ATPase were summarized in Table 1. The concentrations of drugs producing 50% inhibition of Ca^{2+}-Mg^{2+}-ATPase in the presence of modulator were less than those in the absence of modulator.

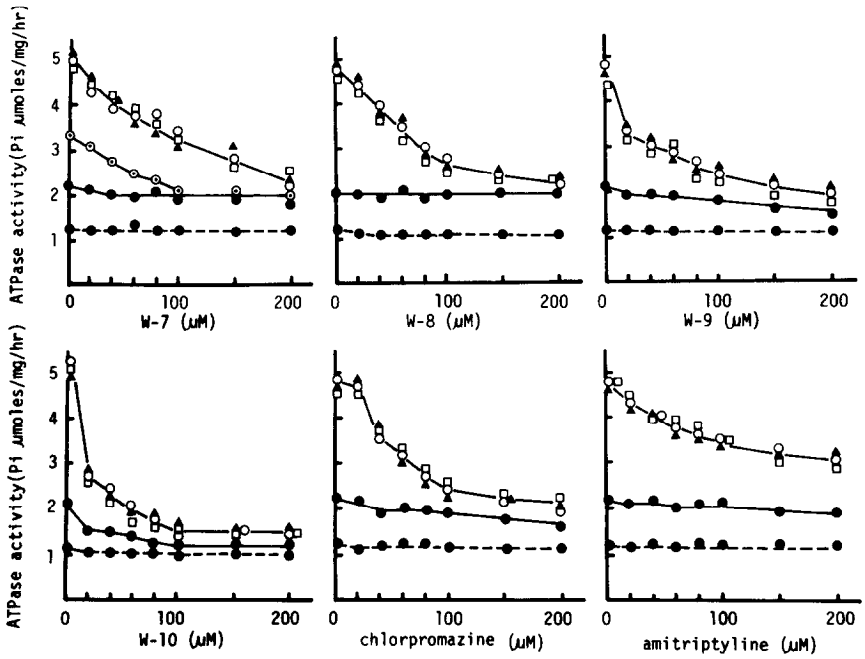


Fig. 1 Effects of modulator interacting agents on erythrocyte ghost ATPase. The ATPase activity was measured in 1.0 ml of incubation mixtures containing 110 μg of erythrocyte ghost proteins and modulator (○——○ 3 μg testis, ▲——▲ 3 μg brain, □——□ 330 μg boiled preparations (see Materials and Methods), dialyzed hemolysate, ⊙——⊙ 15 μg skeletal muscle troponin C, ●——● basal Ca^{2+}-Mg^{2+}-ATPase activity in absence of modulator, ●- - -● Mg^{2+}-ATPase activity). The reaction was started by addition of ATP and carried out at 37°C. Each point is the mean of triplicate determinations. I_{50} (Table 1) is defined as the concentration of drug which produced 50% inhibition of Ca^{2+}-Mg^{2+}-ATPase activation by modulator.

I_{50} values of drugs for testis modulator-activated, brain modulator activated and erythrocyte modulator-activated Ca^{2+}-Mg^{2+}-ATPase were indistinguishable. However, I_{50} of W-7 for troponin C-activated Ca^{2+}-Mg^{2+}-ATPase was lower than that for modulator-activated enzyme activity.

Kinetic analysis: Inhibitory constants of W-7 for testis modulator- and erythrocyte modulator-activated Ca^{2+}-Mg^{2+}-ATPase have been determined by using Dixon plot (Fig. 2). W-7 inhibited these activities in a competitive fashion with a testis and erythrocyte modulator. Ki values of W-7 for testis and erythrocyte modulator were 74 μM and 88 μM respectively. W-7 and several psychotropic drugs also reportedly inhibited phosphodiesterase activity competitively with modulator (10, 14).

Table 1: Effects of several pharmacological agents on erythrocyte ghost ATPase activation by modulator or troponin C.

Inhibitors	Concentration of inhibitor (I_{50} μM)[a]				
	Basal (no modulator)	Testis[b] modulator	Brain[c] modulator	Erythrocyte[d] modulator	Troponin C[e]
N-(aminohexyl)-halogen-naphthalenesulfonamide					
W-7 (5-Cl, 1-R)[f]	2000	108	100	94	64
W-8 (5-Br, 1-R)[f]	2000	62	63	55	
W-9 (5-Br, 2-R)[f]	1200	19	31	19	
W-10 (5-Cl, 2-R)[f]	340	16	15	18	
Chlorpromazine	280	54	52	58	
Amitriptyline	1600	156	156	154	

ATPase activity of an erythrocyte ghost preparation was measured as described in "Materials and Methods".

a. The concentration of the compounds producing 50% inhibition of an erythrocyte ghost Ca^{2+}-Mg^{2+}-ATPase activation by modulator or troponin C. (obtained graphically from data of Fig. 1)

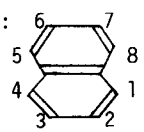
b. 3 μg/tube of rat testis modulator was used as an activator.

c. 3 μg/tube of porcine brain modulator was used as an activator.

d. 330 μg/tube of boiled dialyzed hemolysate was used as an activator.

e. 15 μg/tube of rabbit skeletal muscle troponin C was used as an activator.

f. Structure:



R: $SO_2NH(CH_2)_6NH_2 \cdot HCl$

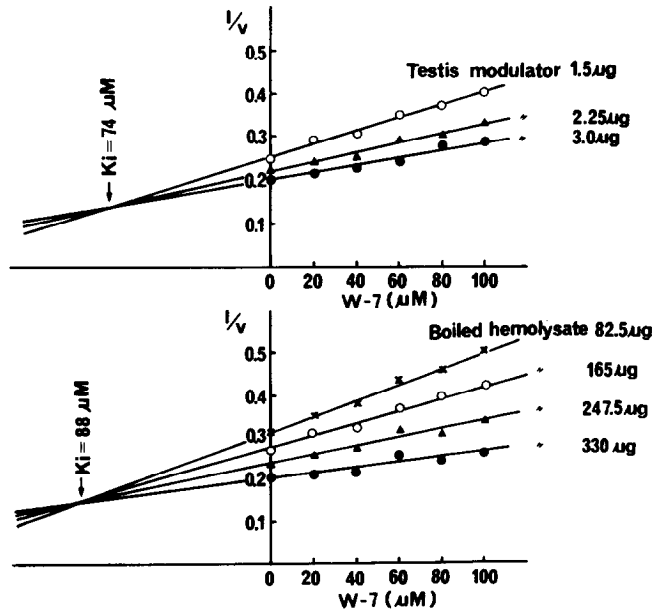


Fig. 2 Kinetic analysis of inhibition of modulator-stimulated Ca^{2+}-Mg^{2+}-ATPase by W-7. ATPase activity was determined in the absence or presence of varying concentration of W-7 in 1.0 ml of incubation mixture containing 110 μg of erythrocyte ghost proteins and varying amounts of testis modulator as indicated in the figure.

Effect of modulator interacting agents on protein kinase activity and erythrocyte hemolysis: We have found that W-7 and psychotropic agents inhibit myosin light chain kinase via Ca^{2+} dependent interaction with modulator (manuscript in preparation). Protein kinase activity of erythrocyte ghosts was examined in the presence or absence of Ca^{2+}, cAMP and testis modulator. A typical experiment was shown in Table 2. W-7 had no effect on protein kinase activity at a concentration of 80 μM which inhibited completely the phosphorylation of chicken gizzard myosin light chain (manuscript in preparation). Effects of W-7, W-8, W-9, W-10, chlorpromazine and amitriptyline on erythrocyte hemolysis were examined.

A typical experiment was shown in Fig. 3. These drugs produced concentration-dependent hemolysis of erythrocytes.

Table 2: Effects of W-7 on erythrocyte ghost protein kinase activity.

Protein kinase activity was measured as described in "Materials and Methods".

Incubations	Exogenous substrate[a]		Endogenous substrate[b]	
	none	+ 80 μM W-7	none	+ 80 μM W-7
EGTA (0.1 mM)	209.4 ± 3.8[c]	215.3 ± 5.5	382.6 ± 8.6	374.5 ± 12.5
EGTA (0.1 mM) + cAMP (2 μM)	491.0 ± 5.9	486.0 ± 12.5	827.2 ± 17.4	824.5 ± 21.2
EGTA (0.1 mM) + testis modulator (3 μg)	230.6 ± 4.0	216.5 ± 10.3	399.3 ± 5.4	390.3 ± 5.8
EGTA (0.1 mM) + $CaCl_2$ (0.2 mM)	121.0 ± 8.0	110.0 ± 6.7	124.8 ± 5.6	120.2 ± 6.6
EGTA (0.1 mM) + $CaCl_2$ (0.2 mM) + cAMP (2 μM)	300.6 ± 11.5	295.4 ± 8.2	342.4 ± 10.2	336.5 ± 7.3
EGTA (0.1 mM) + $CaCl_2$ (0.2 mM) + $^{\text{testis}}_{\text{modulator}}$ (3 μg)	124.5 ± 4.8	128.6 ± 4.8	91.4 ± 3.3	88.5 ± 4.2

a. The incubation tube contained 250 μg histone type II and catalytic amounts (25 μg) of erythrocyte ghost.

b. The incubation tube contained substrate amounts (250 μg) of erythrocyte ghost.

c. Enzyme activity was expressed in picomoles Pi incorporated/10 min (mean ± standard error of triplicate determinations).

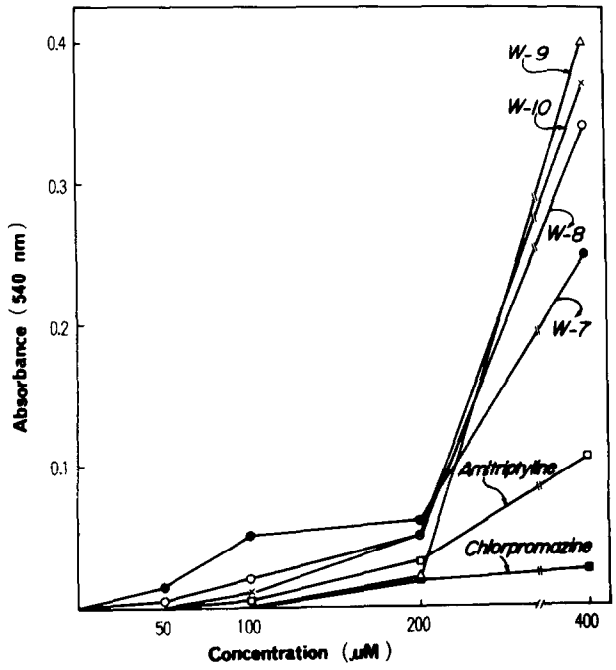


Fig. 3 Effects of modulator interacting agents on erythrocyte hemolysis. Incubations were performed as described in "Materials and Methods".

DISCUSSION

As demonstrated in this paper, modulator interacting agents, W-7, its analogs and some psychotropic drugs inhibit the Ca^{2+}-modulator-complex induced activation of erythrocyte Ca^{2+}-Mg^{2+}-ATPase. It would appear that modulator interacting agents inhibits Ca^{2+}-Mg^{2+}-ATPase by interfering with the formation of protein-protein complex between Ca^{2+}-Mg^{2+}-ATPase and Ca^{2+}-modulator complex. As presented here, I_{50} values of modulator interacting agents for testis modulator-activated-, brain modulator-activated- and erythrocyte activator-activated-ATPase are indistinguishable (Table 1). Furthermore, Ki values of W-7 for testis and erythrocyte modulator-activated ATPase were very similar (Fig. 2). These results provide additional similarities between the modulators from various species and tissues.

The specificity of the modulator interacting agents is shown by the negative results of studies on erythrocyte ghost protein kinase (Table 2) and Mg^{2+}-ATPase (Fig. 1 and Table 1).

As shown in our previous studies (10, 11, 12), modulator interacting agents produce relaxation of isolated vascular strips, inhibit both smooth and skeletal muscle actomyosin ATPase, superprecipitation and inhibit modulator dependent phosphodiesterase. Moreover, we have presented the result that W-7 produces erythrocyte hemolysis. A wide variety of pharmacological actions of modulator interacting agents suggests that modulator is involved in many biological reactions of various cells. The modulator interacting agents provide a very useful tool for elucidating the physiological role of modulator.

REFERENCES

1. Kakiuchi, S., Yamazaki, R., and Nakajima, H. (1970) Proc. Jap. Acad. 46, 589-592.
2. Cheung, W. Y. (1970) Biochem. Biophys. Res. Commun. 38, 533-538.
3. Teshima, Y., and Kakiuchi, S. (1974) Biochem. Biophys. Res. Commun. 56, 489-495.
4. Bond, G. H., and Clough, D. L. (1973) Biochim. Biophys. Acta. 323, 592-599.
5. Luthra, M. G., Hildenbrandt, G. R., and Hanahan, D. J. (1976) Biochim. Biophys. Acta. 419, 164-179.
6. Luthra, M. G., Hildenbrandt, G. R., Kim, H. D., and Hanahan, D. J. (1976) Biochim. Biophys. Acta. 419, 180-186.
7. Jarrett, H. W., and Penniston, J. T. (1977) Biochem. Biophys. Res. Commun. 77, 1210-1216.
8. Gopinath, R. M., and Vincenzi, F. F. (1977) Biochem. Biophys. Res. Commun. 77, 1203-1209.
9. Jarrett, H. W., and Penniston, J. T. (1978) J. Biol. Chem. 253, 4676-4682.
10. Hidaka, H., Yamaki, T., Asano, M., and Totsuka, T. (1978) Blood Vessels. 15, 55-64.
11. Hidaka, H., Yamaki, T., Totsuka, T., and Asano, M. (1979) Mol. Pharmacol. 15, 49-59.
12. Hidaka, H., Asano, M., Iwadare, S., Matsumoto, I., Totsuka, T., and Aoki, N. (1978) J. Pharmacol. Exp. Ther. 207, 8-15.
13. Honda, F., Katsuki, S., and Sakai, N. (1973) Jap. J. Pharmacol. 23, suppl. 27.
14. Levin, R. M., and Weiss, B. (1976) Mol. Pharmacol. 12, 581-589.
15. Fiske, C. H., and Subbarow, Y. (1925) J. Biol. Chem. 66, 375-400.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randoll, R. J. (1951) J. Biol. Chem. 193, 265-275.
17. Kobayashi, R., and Field, J. B. (1978) Biochim. Biophys. Acta. 539, 411-419.
18. Ebashi, S., Wakabayashi, T., and Ebashi, F. (1971) J. Biochem. 69, 441-445.
19. Perry, S. V., and Cole, H. A. (1974) Biochem. J. 141, 733-743.
20. Hosey, M. M., and Tao, M. (1976) Biochemistry. 15, 1561-1568.