- activated ATPase. A possible target of aldosterone. *J. clin. Invest.* **55**, 655-660 (1975)
- 8) WESTENFELDER, C., AREVALO, G.J., BARANOWSKI, R.L., KURTZMAN, N.A. AND KATZ, A.I.: Relationship between mineralocorticoids and renal Na, K-ATPase; sodium reabsorption. *Am. J. Physiol.* **233**, F593-F599 (1977)

DIFFERENCES IN SPEED AND DEGREE OF MYOSIN B SUPERPRECIPITATION IN THE PRESENCE OF DIVALENT CATIONS

Michio YAJIMA and Kazumi TAKEYA

*Department of Pharmacology, Aichi Medical University,
Nagakute, Aichi 480-11, Japan*

Accepted October 2, 1978

Efforts have been made to acquire precise quantitative evaluation for the superprecipitation of contractile protein myosin B. Ebashi first reported a method for evaluating the degree of superprecipitation by turbidimetry at 660 m μ (1). Watanabe used two parameters for study of superprecipitation; the time required to reach the half maximum increase in turbidity ($t_{0.5}$) and the maximum increase in turbidity* (ΔOD , extent) (2). In the present experiments, we introduced the velocity of superprecipitation at $t_{0.5}$ ($V_{t_{0.5}}$) in addition to the above two parameters (Fig. 1). The objective was to obtain information concerning the effects of Ca, Mg and Mn on these three parameters.

Myosin B was extracted from the leg muscle of rabbit (1). The superprecipitation of myosin B was continuously measured using a spectrophotometer at 660 m μ (Hitachi 100-01) and the findings were displayed on paper (National pen recorder VP-654B). The reaction mixture in a total volume of 3.0 ml, contained 0.6 mg/ml myosin B, 0.06 M Tris-maleate

FIG. 1. Parameters in time course of the superprecipitation of myosin B. Parameters ($t_{0.5}$, F_{max} and $V_{t_{0.5}}$) are illustrated on the curve in case of b. Three representative cases in the time course of superprecipitation are shown: a, control (no added divalent cations); b, presence of 10^{-4} M $MnCl_2$; c, presence of 10^{-4} M $MgCl_2$.

* We termed this parameter F_{max} .

buffer (pH 6.8) and varying amounts of divalent cations (CaCl_2 , MgCl_2 and MnCl_2). The reaction was started by addition of 0.5 ml of 6 mM ATP- Na_2 (final conc. 1 mM). To avoid fluctuation of the results, special attention was paid to the adjustment of ionic strength with KCl solution (0.9 M) at 0.17 and to maintain the temperature of the mixture at $26 \pm 0.5^\circ\text{C}$. The endogenous Ca and Mg contents of the reaction mixture were estimated by atomic absorption spectroscopy at about 8 and 13 μM , respectively. The concentrations of ionic form of these metals were presumably at a concentration below 1 μM , considering that the effects on $t_{0.5}$ were significant at $p < 0.05$ (Fig. 2a).

A time point of the onset of superprecipitation was delayed by both Mg and Mn (Fig. 1). Time required to reach half maximum increase in turbidity of myosin B with Mg was markedly longer than that with Mn; i.e., the values of $t_{0.5}$ in the presence of 10^{-4} M of either MgCl_2 or MnCl_2 were 135 ± 6.9 min and 39 ± 0.9 min, respectively. Calcium produced a dose-dependent shortening of $t_{0.5}$ and this effect was more pronounced in the presence of Mg than that of Mn (Fig. 2a). The level of maximum increase in turbidity of superprecipitation (F_{max}) was observed to be elevated by the presence of Mn about 30% over the control value. Electromicroscopic examinations, however, showed no special findings in the ultrastructure of the superprecipitated myosin B (unpublished data). Calcium in a low concentration (10^{-6} – 10^{-4} M) did not affect the superprecipitation, while, a higher concentration (10^{-3} – 10^{-2} M) produced an increase in F_{max} in the presence of Mg or Mn (Fig. 2b). The velocity of superprecipitation at $t_{0.5}$ in the control experiment was as fast as 0.70 ($\Delta\text{OD}/\text{min}$), whereas in the presence of Mg or Mn (10^{-4} M) the velocity slowed down to 0.008 or to 0.042. Between Ca and other divalent cations an antagonistic relation was found in $V_{t_{0.5}}$ (Fig. 2c); the velocity decreased by Mg (10^{-4} M) was restored by adding Ca in a dose-dependent manner,

FIG. 2. Effects of Mg and Mn on the parameters of superprecipitation under varied CaCl_2 concentrations. Degree of superprecipitation was evaluated by change in turbidity of myosin B. a, $t_{0.5}$: time required to reach the half maximum increase in turbidity; b, F_{max} : the maximum increase in turbidity; c, $V_{t_{0.5}}$: velocity of superprecipitation at $t_{0.5}$. Each point and bar represent mean $\pm$ S.E. ($N=3$). $\circ$ — $\circ$: 10^{-4} M MgCl_2 . $\bullet$ — $\bullet$: 10^{-4} M MnCl_2 . C_1 : no added divalent cations such as Mn, Mg and Ca. C_2 : no added CaCl_2 .

e.g., CaCl_2 of 10^{-4} , 10^{-3} and 10^{-2} M increased the velocity by an average of 2.7, 4.5 and 8.3 times as compared with the control in the presence of Mg. The suppressive effect of Mg on $V_{t_{0.5}}$ developed concentration-dependently in the range tested from 10^{-6} M to 10^{-4} M (data not shown). These results indicate that the speed of superprecipitation is regulated by Ca and Mg in an antagonistic relation.

In comparison with Mg and Mn at an equimolar basis of 10^{-4} M, Mg suppressed $V_{t_{0.5}}$ with a potency 5 times greater than Mn. Calcium also accelerated the onset ($t_{0.5}$) and velocity ($V_{t_{0.5}}$) of the superprecipitation of myosin B in the presence of Mn; e.g., $V_{t_{0.5}}$ was accelerated twice by the addition of 10^{-3} M CaCl_2 , however, Mn inhibited $V_{t_{0.5}}$ concentration-independently in the range from 10^{-6} – 10^{-2} M (data not shown). This prolongating effect on $t_{0.5}$ is presumably in accord with the results in reports that clearing response on superprecipitation or inhibitory effect on ATPase activity of myosin B was strengthened by Mn as well as Mg (3, 4). The mechanism of increased $F_{\max}$ by Mn is obscure from the present experiments, however, the paradoxical positive inotropic effect of Mn on isolated guinea-pig myocardium has been reported (5). It has also been reported that Mn blocked Ca influx in various excitable cells (6, 7, 8). Moreover, the evidence that Mn carried transmembrane inward current suggests that Mn directly affects the contractile protein complex (9, 10, 11). The inhibitory effect of Mn on $t_{0.5}$ or $V_{t_{0.5}}$ would explain the negative inotropic effect of Mn in the early stage (12).

In summary, a new parameter $V_{t_{0.5}}$, speed of superprecipitation is introduced in addition to $t_{0.5}$ and $F_{\max}$ (2). On these three parameters of superprecipitation, the influence of divalent cations, Ca, Mg and Mn was analyzed.

REFERENCES

- 1) EBASHI, S.: Calcium binding activity of vesicular relaxing factor. *J. Biochem.* **50**, 236–244 (1961)
- 2) WATANABE, S.: Relation between superprecipitation and ATPase of myosin B. *J. Biochem.* **68**, 913–921 (1970)
- 3) FUJII, T. AND MARUYAMA, K.: Manganese-induced clearing response of actomyosin at low temperatures. *Sci. Pap. Coll. gen. Educ., Univ. Tokyo* **20**, 163–169 (1970)
- 4) ISHIKAWA, Y. AND MARUYAMA, K.: Effect of metal ions on the ATPase activity of natural actomyosin. *Zool. Mag.* **73**, 213–214 (1964)
- 5) VIERING, M.: Positive inotropic effect of manganese in the guinea-pig myocardium. *Arch. Pharmacol.* **297**, R19 (1977)
- 6) HAGIWARA, S.: Membrane properties of the barnacle muscle fiber. *Ann. N. Y. Acad. Sci.* **137**, 1015–1024 (1966)
- 7) BAKER, P., MEVES, H. AND RIDGWAY, E.B.: Effects of manganese and other agents on the calcium uptake that follows depolarization of squid axons. *J. Physiol.* **231**, 511–526 (1973)
- 8) KOHLHARDT, M., BAUER, B., KRAUSE, H. AND FLECKENSTEIN, A.: Selective inhibition of the transmembrane Ca conductivity of mammalian myocardial fibers by Ni, Co and Mn ions. *Pflügers Arch.* **338**, 115–123 (1973)
- 9) OCHI, R.: Manganese-dependent propagated action potentials and their depression by electrical stimulation in guinea-pig myocardium perfused by sodium-free media. *J. Physiol.* **263**, 139–156 (1976)
- 10) DELAHAYES, J.F.: Depolarization-induced movement of Mn^{2+} across the cell membrane in the guinea pig myocardium. *Circulation Res.* **36**, 713–718 (1975)

- 11) FUKUDA, J. AND KAWA, K.: Permeation of manganese, cadmium, zinc and beryllium through calcium channels of an insect muscle membrane. *Science* **196**, 309-311 (1977)
- 12) TAKEYA, K. AND REITER, M.: Effect of divalent manganese ions on action potential and contractility of cardiac muscle. *Arch. Pharmacol.* **275**, 213-226 (1972)

EFFECTS OF d,l-PROPRANOLOL AND OTHER BETA ADRENERGIC RECEPTOR BLOCKING AGENTS ON MEMBRANE FLUIDITY OF ERYTHROCYTES

Shin-ichi AKIYAMA* and Hideki IGISU**

*Kyushu Cancer Center, 595 Notame, Minami-ku, Fukuoka 815,
and **Institute of Health Science and Department of Neurology, Neurological
Institute, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

Accepted October 18, 1978

In addition to the beta adrenergic receptor blocking action, propranolol has been known to be lipid soluble with local anesthetic properties (1). The local anesthetic activity of propranolol may account for the antiarrhythmic effects of the drug (2). It was reported that propranolol might change the structural state of membrane phospholipids of erythrocytes (3). We studied the effects of d,l-propranolol and other beta adrenergic receptor antagonists, practolol and pindolol, on the fluorescence polarization of DPH embedded in human erythrocyte membranes.

Erythrocyte membranes were prepared from human blood by hypotonic lysis (4). After discarding the serum and buffy coat carefully, red blood cells were washed 3 times with 0.85% NaCl and hemolysed in 10 mM Tris-HCl (pH 7.4). The ghosts were suspended and centrifuged 5 times in this buffer at 20000 g for 15 min.. The membranes were stored at -80°C until use. Fluorescence labeling of the erythrocyte membranes was done as described by Shinitzky and Inbar (5). 1,6-Diphenyl 1,3,5-hexatriene (DPH), which has been established as a fluidity probe for hydrocarbon regions of biological membranes and liposomes, was used (6). One volume of membrane suspension with the protein concentration of 0.4 mg/ml was mixed with the same volume of labeling solution; 2×10^{-6} M DPH dispersion in 10 mM Tris-HCl (pH 7.4) which was freshly prepared by diluting a stock solution of 2×10^{-3} M DPH in tetrahydrofuran. After thorough mixture and incubation for 1 hour at 25°C , the labeled membranes were washed twice with 10 mM Tris-HCl buffer (pH 7.4), resuspended in the same buffer to make the protein concentration 0.3 mg/ml and used in the experiments. Measurements of the intensity of the fluorescence were made using a Hitachi Spectrofluorometer, Model MPF-4 (Hitachi, Ltd., Tokyo, Japan), equipped with polarizers and a thermostatic cell holder. Excitation was at 366 nm with a band pass of 10 nm and emission was measured at 427 nm (emission maximum) after passing through

* Present address: Department of Biochemistry, Oita Medical School, Oita 870, Japan