INCREASE IN Ca\(^{2+}\) (Mg\(^{2+}\))-ATPase ACTIVITY INDUCED BY A MOLSIDOMINE DERIVATIVE (SIN-1 A) AND NITROGLYCERIN IN MICROSONAL FRACTION OF GUINEA-PIG THORACIC AORTA

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The amount of phosphoric acid liberated from ATP by Ca\(^{2+}\) (Mg\(^{2+}\))-ATPase in microsomal fraction of guinea-pig thoracic aorta decreased with decreasing concentrations of calcium ions from 20.0 to 2.5 mM in the mixture of the enzyme and substrate. When CaCl\(_2\) (2.5 mM) and MgCl\(_2\) (5.0 mM) were present in the substrate, both nitroglycerin (0.1 to 1.0 mM) and SIN-1 A (a molsidomine derivative, 0.05 to 1.0 mM) increased the liberated phosphoric acid in a concentration-dependent manner. The contractile tension of smooth muscle prepared from guinea-pig thoracic aorta, which was previously increased by the pretreatment with prostaglandin F\(_{2\alpha}\) (5.0 \(\mu\)M), was relaxed by both nitroglycerin and SIN-1 A (0.01 to 100 \(\mu\)M each) in a concentration-dependent manner. From the results, it is assumed that the stimulation of Ca\(^{2+}\) (Mg\(^{2+}\))-ATPase [Ca\(^{2+}\)-pump ATPase] activity induced by nitroglycerin and SIN-1 A in the microsome of thoracic aorta takes part in the relaxation of contractile tension in the tissue.

In determining the action mechanism of muscle relaxants such as nitroprusside and nitroglycerin, it has been suggested\(^1\)\(^{-}\)\(^7\) that the action is mediated by an increase in the tissue level of cyclic GMP. Research by Diamond et al.\(^8\)\(^,\)\(^9\) raises doubt regarding this hypothesis, and the action mechanism of the drugs remains unclear. It is well known that the increase in activity of Ca\(^{2+}\)-activated and Mg\(^{2+}\)-dependent ATPase [Ca\(^{2+}\) (Mg\(^{2+}\))-ATPase], i.e., Ca\(^{2+}\)-pump ATPase relates to muscle relaxation. However, there are few reports on drugs which directly stimulate the enzyme activity, although Vincenzi and Ashelman\(^10\) demonstrated that olate and sodium dodecylsulfate as well as calmodulin stimulate the enzyme activity.

In this study, we investigated the effects of nitroglycerin and a molsidomine derivative (SIN-1 A) on the enzyme activity in the microsomal fraction of guinea-pig thoracic aorta at various concentrations of calcium ions. In addition, the effects of the two drugs on contractile tension of the aortic smooth muscle were examined. SIN-1 A (N-nitroso-N-morpholinoaminoacetanitride) is an active metabolite of molsidomine (N-ethoxycarbonyl-3-morpholinosydnonimine, SIN-10)\(^11,\)\(^12\) which was donated by Takeda Chemical Ind. Co., Ltd., Nitroglycerin was donated by Nihon-kayaku Co., Ltd.

MATERIALS AND METHODS

Preparation of Microsomal Fraction Containing ATPase

Guinea-pigs of both sexes weighing 450 to

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Key Words:
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Fig.1. The change of Ca\textsuperscript{2+} (Mg\textsuperscript{2+})-ATPase activity induced by decreasing CaCl\textsubscript{2} (●—●) and the influence of nitroglycerin (1.0 mM) (○—○) and SIN-1 A (1.0 mM) (○—○) on the enzyme activity in microsomal fraction of guinea-pig thoracic aorta. The enzyme activity is expressed by the amount of phosphoric acid liberated from ATP by the enzyme which is contained in the protein (1.0 mg) of fraction extracted from the tissue for one hour (µM/mg protein/hr). In the reaction mixture of the substrate and enzyme, MgCl\textsubscript{2} 5.0 mM, CaCl\textsubscript{2} 20.0 mM, NaCl 50.0 mM, tris-acetate 50.0 mM and ATP 5.0 mM were present. When the CaCl\textsubscript{2} was decreased from 20.0 mM to 10.0, 5.0 and 2.5 mM, NaCl 15.0, 22.5 and 26.3 mM were added to adjust the osmotic pressure. At a high concentration of CaCl\textsubscript{2} (20.0 mM), the increase in the enzyme activity induced by each 1.0 mM of nitroglycerin and SIN-1 A was slight. Each point and standard error is calculated from values obtained in 8 preparations.

500 g were used. The unanaesthetized animal was killed by a head blow and the thoracic aorta (0.40 ± 0.05 g, n = 10) was removed. The tissue was immersed in a cold water solution, 10 ml of which consisted of 9.65 ml of sucrose (250 mM), 0.3 ml of EDTA-3 Na (10.0 mM) and 0.05 ml of sodium deoxycholate (50.0 mM) in distilled water. The water solution was kept at 3 to 4°C and 10 ml was used for each gram of the removed wet tissue. The tissue was dissected using a polytron (Kinematica) and homogenized in a glass-Teflon homogenizer. The homogenate was centrifuged using an automatic high speed refrigerate centrifuge (Hitachi, 20 PR-2) for 15 min (700 G). The supernate was recentrifuged in the same instrument for 20 min (7,000 G) to precipitate the mitochondria. The supernate was then ultracentrifuged using an automatic preparative ultracentrifuge (Hitachi, 65 P-1) for 60 min (100,000 G) to precipitate the microsome. The precipitate was mixed with 4.0 ml of sucrose solution (250 mM) and homogenized in the glass-Teflon homogenizer. The resulting homogenate was assumed to contain various kinds of ATPases.

**Determination of Protein Content**

The protein content in the microsomal fraction was determined using bovine serum albumin as a standard, according to Lowry's method.\textsuperscript{13} The total protein in the fraction obtained from one gram of the tissue of thoracic aorta was calculated to be 6.0 to 7.0 mg. To confirm that the fraction contained no mitochondria, succinate dehydrogenase was used as a marker enzyme according to a modified method of King.\textsuperscript{14}
**Assay of ATPase Activity**

Assay of ATPase activity in the microsomal fraction obtained was performed according to a modified method of Fiske & Subbarow\textsuperscript{15} or Berenblum & Chain\textsuperscript{16}. The substrate used (0.9 ml) was adjusted to consist of

- (a) 0.1 ml of inorganic ion solution containing MgCl\(_2\) 50.0 mM, CaCl\(_2\) 200 mM and NaCl 500 mM in water,
- (b) 0.5 ml of tris-acetate buffer solution (0.1 M, pH: 7.45),
- (c) 0.2 ml of distilled water (reserved for the tested drug), and
- (d) 0.1 ml of ATP solution (50.0 mM).

The enzyme (0.1 ml) was adjusted to contain 0.05 mg of protein in 0.1 ml of sucrose solution (250 mM). The sum of the substrate and enzyme was 1.0 ml and the concentration of the added inorganic ions and drugs following in the text is expressed as a concentration in the mixture, i.e., as a final concentration. When the substrate was practically reacted by the enzyme, (a), (b), enzyme, (c) and (d) were mixed one by one, reacted at 37°C for 30 min, and immediately thereafter cooled to 0°C. After adding 0.5 ml of trichloracetic acid (600 mM), 1.0 ml of sodium molybdate (100 mM) and 1.0 ml of 1.5 N H\(_2\)SO\(_4\), the solution appeared yellow. The solution, then, was mixed with 4.0 ml of isobutanol to transfer the yellow colored phosphomolybdate into isobutanol layer, which was then removed to another tube. To the isobutanol layer (2.0 ml), 2.0 ml of 0.05% (w/v) KHSO\(_4\) containing 0.5% (w/v) of ascorbic acid and 1.0 ml of ethanol were added and reacted at 37°C for 30 min. The color changed from yellow to blue, and the blue absorption at 700 nm was measured by using a spectrophotometer (Hitachi 340-type), from which the amount of liberated phosphoric acid (Pi \(\mu\)M/mg protein/hr) was calculated.

**Contractile Tension in Smooth Muscle Strip of Thoracic Aorta**

The thoracic aorta isolated from the guinea-pig was helically cut at an angle of approximately 45° to the longitudinal axis, into a strip.
RESULTS

In the presence of MgCl₂ (5.0 mM), the concentration of CaCl₂ in the substrate was decreased from 20.0 to 2.5 mM while adjusting the osmotic pressure with NaCl. The result was that the phosphoric acid liberated from ATP by the enzyme decreased with decreasing CaCl₂. When the change of osmotic pressure produced by the decrease of CaCl₂ was not adjusted with NaCl, the value of Pi was by 2 to 6% smaller than those in the cases in which the osmotic pressure was adjusted. When the concentration of CaCl₂ was further decreased to 1.0, 0.1 or 0.01 mM, adjusting the osmotic pressure with or without NaCl, the value of Pi was very variable and the standard error of the mean value was very large. When the MgCl₂ was removed from the substrate, the value of Pi was by 10 to 30% larger than that in the case in which the MgCl₂ was present.

When MgCl₂ (5.0 mM) was present and CaCl₂ concentration was decreased from 20.0 to 2.5 mM in the substrate adjusting the osmotic...
pressure with or without NaCl, the addition of strophanning-G (0.1 to 1.0 mM), which is a characteristic inhibitor of Na\(^+\), K\(^+\)-ATPase activity, did not influence the value of Pi. When the strophanning-G was replaced by SIN-1 A or nitroglycerin (1.0 mM each), the drugs increased the value of Pi: the increase induced by the drugs was more remarkable in the cases in which the lower concentrations of CaCl\(_2\) were present. When CaCl\(_2\) (2.5 mM) and MgCl\(_2\) (5.0 mM) were present with NaCl used to adjust the osmotic pressure, the increase induced by SIN-1 A (1.0 mM) in the value of Pi was 33.2 ± 5.0% (n = 8) and that induced by nitroglycerin (1.0 mM) was 20.1 ± 4.6% (n = 8). When the CaCl\(_2\) (2.5 mM) was replaced by CaCl\(_2\) (20.0 mM), both SIN-1 A and nitroglycerin (1.0 mM each) increased the value of Pi, though slightly (Fig. 1). When the change of osmotic pressure produced by decreasing CaCl\(_2\) was not adjusted with NaCl, the percent increases in enzyme activity induced by the drugs were almost the same as those in the cases in which the NaCl was used, although both value of Pi in control and the drug effects were by 2 to 6% smaller. When MgCl\(_2\) was removed from the substrate but CaCl\(_2\) (20.0 to 2.5 mM) was present, or when CaCl\(_2\) was removed but MgCl\(_2\) (0.5 to 5.0 mM) was present, the SIN-1 A and nitroglycerin (1.0 mM each) less or hardly influenced the value of Pi.

When the MgCl\(_2\) (5.0 mM) and CaCl\(_2\) (2.5 mM) were added to the substrate while adjusting the osmotic pressure with NaCl, the SIN-1 A (0.05 to 1.0 mM) or nitroglycerin (0.1 to 1.0 mM) increased the value of Pi in a concentration-dependent manner (Fig. 2). The percent increases in Pi induced by the drugs (1.0 mM each) are described above.

The contractile tension of the strip of isolated guinea-pig thoracic aorta, which had been previously contracted by prostaglandin F\(_{2\alpha}\) (0.5 μM), was relaxed by both SIN-1 A and nitroglycerin (0.01 to 100 μM each) in a concentration-dependent manner: SIN-1 A (100 μM) decreased the tension by 96.6 ± 2.3% (n = 8) and nitroglycerin (100 μM) by 90.2 ± 0.9% (n = 8) (Fig. 3).

**DISCUSSION**

The microsomal fraction extracted from the thoracic aorta in the present work is assumed to contain various kinds of ATPases. No contamination of the extracted fraction with mitochondria was confirmed by use of a modified method of King.\(^{14}\) The constituents of the substrate used in the reaction with the ATPase suggest that the enzyme involved in the liberation of phosphoric acid from ATP was Ca\(^{2+}\)-activated and Mg\(^{2+}\)-dependent ATPase \([\text{Ca}^{2+}(\text{Mg}^{2+})\text{-ATPase}], \) i.e., Ca\(^{2+}\)-pump ATPase. The concentration of calcium ion, at which the ATPase activity is highest, was 20.0 mM. This concentration of calcium ion approximated those which were used by Sorensen et al.\(^{17}\) to activate the enzyme although the tissues were different. The minor influence of strophanning-G on the amount of phosphoric acid liberated from ATP suggests that Na\(^+\), K\(^+\)-ATPase in the microsomal fraction is scarcely involved in the liberation of phosphoric acid.

The Ca\(^{2+}\)(Mg\(^{2+}\)))-ATPase activity decreased when the calcium ions in the substrate were reduced. At sufficient concentrations of calcium ions, the effects of SIN-1 A and nitroglycerin on the enzyme activity were not as marked. The sufficient increase in the activity was already induced by the sufficient calcium ions, while the effects were marked at insufficient calcium ions. In the physiological experiment to examine the drug effects on the contractile tension of thoracic aorta, both drugs showed a relaxing effect on the smooth muscle. The results suggest that the stimulation of Ca\(^{2+}\)-pump ATPase activity in the microsome induced by the both drugs is involved in the relaxation of contractile tension in the muscle. However, the percentage of increase in the enzyme activity that induces smooth muscle relaxation has not yet been determined.

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