Effects of Protopine on Blood Platelet Aggregation. II.[1] Effect on Metabolic System of Adenosine 3′,5′-Cyclic Monophosphate in Platelets

Hidemi SHIOMOTO,* Hideaki MATSUDA and Michinori KUBO

Faculty of Pharmaceutical Sciences, Kinki University, 3–4–1, Kowakae, Higashiosaka, Osaka 577, Japan. *Received March 20, 1990*

The mode of action of protopine on rabbit platelet aggregation was investigated in the metabolic system of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) *in vitro* experimental models.

The inhibitory activity of protopine on adenosine 5′-diphosphate induced platelet aggregation was increased in the presence of prostaglandin I_2 or papaverine in platelets. Protopine elevated content of the basal cyclic AMP accumulation in platelets and enhanced activity of crude adenylate cyclase prepared from platelets, but was ineffective on cyclic AMP phosphodiesterase.

It is concluded that protopine has an inhibitory activity on platelet aggregation, activates adenylate cyclase and increases cyclic AMP content in platelets, in addition to other inhibitory actions in the metabolic system of cyclic AMP.

Keywords Corydalis tuber; protopine; platelet aggregation; cyclic AMP; adenylate cyclase

In a series of pharmacological studies on Corydalis tuber, we reported that a kind of alkaloidal component, protopine isolated from a methanolic extract of the tuber showed an inhibitory effect on *in vitro*, *ex vivo* and *in vivo* platelet aggregation.[1,2] Since the mode of action of protopine against the platelet aggregation has not been reported, the present investigation studied the effect of this component on the metabolic system of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) in platelets.

Experimental

Materials Protopine was isolated from Corydalis tuber by the method of Kaneko and Naruto.[3] Sources of the materials were as follows: adenosine 5′-diphosphate sodium salt (ADP), disodium phosphocreatine and creatine phosphokinase (from rabbit muscle, Type I, 185 unit/mg protein, Sigma Chemical Co., U.S.A.), prostaglandin I_2 (PGI_2, Funakoshi Pharmaceuticals Co., Ltd., Japan), adenosine 5′-triphosphate disodium salt (ATP), adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and papaverine hydrochloride (Nacalai Tesque, Inc., Japan).

Animals Male Kwl : JW strain rabbits (2.0—2.5 kg) were used for the experiments. They were maintained in an air-conditioned room with lighting from 7 a.m. to 7 p.m. The room temperature (about 23 °C) and humidity (about 60%) were controlled automatically. A laboratory pellet chow (Labo R Stock, Nihon Nosan Kogyo K.K., Japan) and water were given freely.

Preparation of Platelet-Rich or -Poor Plasma Whole blood samples were collected from the heart of pentobarbital-anesthetized rabbits. Nine ml of the blood and 1 ml of sodium citrate (3.8%) were transferred into a plastic tube, and centrifuged at 200 *g* at room temperature for 10 min to obtain platelet-rich plasma (PRP, 3.0×10^8 platelets/ml). PRP was removed with a siliconized pipet, and stored in a plastic tube. The remaining red cell precipitate of the blood samples was further centrifuged at 1800 *g* for 30 min to give platelet-poor plasma (PPP). PRP and PPP were used for assay of platelet aggregation.

Preparation of Washed Platelet Suspension The citrated PRP was resuspended in 25 mM Tris–HCl buffer (pH 6.8) containing 130 mM NaCl, 0.1% glucose and 1.5 mM ethylenediaminetetraacetic acid (EDTA), and the suspension was centrifuged at 1800 *g* at 4 °C for 10 min, then rinsed twice with the same solution as used for the resuspension. The pellet was finally suspended in HEPES buffer (pH 7.35) [140 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 0.1% glucose, 0.35% bovine serum albumin (BSA), 3.75 mM NaH_2PO_4, 5.0 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)]. This final suspension (5.0×10^8 platelets/ml) was referred to as washed platelet suspension (WPS).

Assay of Platelet Aggregation The assay of platelet aggregation was performed according to the method of Born and Cross.[4] A 190 μl aliquot of PRP was placed in a test tube and the content was stirred at 1200 rpm for 2 min at 37 °C, then a 5 μl aliquot of a test solution was added and incubated for 10—300 s; an aggregating agent, ADP (50.0 μM) was then added to the reaction mixture. Changes in the light transmittance of the reaction mixture were continuously recorded with a Husm system platelet aggregometer (Rika Electric Co., Japan) and the transmission at the maximal aggregation after the addition of an aggregating agent was recorded. Platelet aggregation was expressed as the percent increase in the transmittance taking the transmittance of a control solvent (dimethylsulfoxide, DMSO) as zero.

Combined effect of protopine and PGI_2 or papaverine on *in vitro* platelet aggregation was determined as mentioned above. The concentration of PGI_2 (2.5 nM) or papaverine (0.1 mM) was found to give approximately 5% of the required inhibitory rate on ADP (50.0 μM)-induced platelet aggregation in PRP.

Determination of Cyclic AMP Content Content of cyclic AMP was measured in 300 μl aliquots of WPS incubated at 37 °C for 2 min with 100 μl test solution (in 10% DMSO/HEPES buffer). Incubations were terminated by addition of 400 μl of ice-cold 30% w/v trichloroacetic acid (TCA)/0.1 N HCl. The platelets were then sonicated at 4 °C for 30 s by a sonifier cell disruptor (Branson, U.S.A.). Precipitated proteins were removed by centrifugation and the supernatant solution was washed 5 times with water-saturated ethylether in order to remove the TCA. Trace amount of ethylether remaining in the supernatant was removed by placing the tube in a warm water bath for a few minutes. The solution was then assayed for cyclic AMP. Cyclic AMP was radioimmunochemically determined using a cyclic AMP assay kit (Yamasa Shoyu Co., Japan).

Also, cyclic AMP was measured in 300 μl aliquots of WPS preincubated with 50 μl of papaverine (0.5 mM, in 10% DMSO/HEPES buffer) at 37 °C for 2 min and incubated with 50 μl of test solution (in 10% DMSO/HEPES buffer).

Assay of Platelet Adenylate Cyclase Activity A modification of the method of Haslam and Lynham[5] was used. The platelet pellet described above was resuspended in 25 mM Tris–HCl buffer (pH 7.4) containing 130 mM NaCl. The washed platelet suspension (1.0×10^9 platelets/ml) was rapidly frozen in a dry-ice acetone bath and thawed at 37 °C. The resulting lysate was centrifuged at 38000 *g* for 20 min at 4 °C. The pellet, which was referred to as platelet membrane fraction, was resuspended in 25 mM Tris–HCl buffer (pH 7.5) containing 130 mM NaCl. This suspension containing 0.66 mg protein/ml was stored at 0 °C until use for assay of adenylate cyclase activity. The mixture of 50 μl of the platelet membrane fraction and the 50 μl of test solution (in 5% DMSO/the 25 mM Tris–HCl buffer) was preincubated at 30 °C for 5 min. Following the incubation, 200 μl of 25 mM Tris–HCl (pH 7.5) containing 0.4 mM ATP, 5.0 mM $MgCl_2$, 4.0 mM disodium phosphocreatine, 40 units creatine phosphokinase/ml, 1.0 mg BSA/ml and 0.25 mM papaverine were added. The mixture was incubated at 30 °C for 20 min and the reaction was stopped by rapid heating in a boiling water bath for 2 min. The mixture was then centrifuged at 1800 *g* for 20 min, and the supernatant solution was assayed for cyclic AMP. Platelet adenylate cyclase activity was expressed as the production (pM/mg protein/min) of cyclic AMP from ATP.

Assay of Platelet Cyclic AMP Phosphodiesterase Activity The platelet pellet described above was resuspended in 25 mM Tris–HCl buffer (pH 7.5) containing 130 mM NaCl and 1.0 mM $MgCl_2$. The washed platelet suspension (1.0×10^9 platelets/ml) was sonicated at 4 °C for 2 min by the sonifier cell disruptor and a soluble phosphodiesterase preparation was obtained from the sonicated homogenate by centrifugation at 100000 *g* at 4 °C for 60 min. This supernatant containing 0.40 mg protein/ml was

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referred to as phosphodiesterase preparation. Cyclic AMP phosphodiesterase activity was measured by the modified method of Hidaka and Asano.[6] A mixture of 200 μl of the phosphodiesterase preparation and 50 μl of the test solution (in 5% DMSO/the 25 mM Tris–HCl buffer) was preincubated at 30 °C for 5 min. To the mixture incubated was added 250 μl of 25 mM Tris–HCl buffer (pH 7.5) containing 1.0 μM cyclic AMP and 5.0 mM $MgCl_2$ and then incubated at 30 °C for 10 min. The reaction was stopped by rapid heating in a boiling water bath for 2 min. Cyclic AMP was isolated from other nucleotides by the addition of 500 μl of 0.25 M $ZnSO_4$ to the reaction mixture, followed by 500 μl of 0.25 M $Ba(OH)_2$ according to the method of Krishna *et al.*[7] The precipitate was separated by centrifugation, and the cyclic AMP of the supernatant was determined by radioimmunoassay. Platelet cyclic AMP phosphodiesterase activity was expressed as the hydrolyzed cyclic AMP (nM/mg protein/min).

Determination of Protein Protein was determined according to the method of Lowry *et al.*[8]

Statistical Analysis The experimental data were tested for statistically significant differences by means of Student's *t* test.

Results

Platelet Aggregation The effect of protopine on platelet aggregation is shown in Fig. 1. ADP-induced platelet aggregation was maximally inhibited after a 60-s incubation with 0.1, 0.25 or 1.0 mM of protopine.

Combined Effect on Platelet Aggregation In the experiment shown in Fig. 2, protopine was tested as an inhibitor of aggregation with and without the addition of PGI_2 or papaverine (2.5 nM, 0.1 mM, respectively, concentrations that alone had little effect on aggregation). Protopine by itself, added 60 s before ADP, caused an inhibition of aggregation with an S-shaped log (dose)–response curve at concentrations of 0.01—1.0 mM. In the presence of PGI_2 or papaverine the dose–response curve was unaltered in shape but was shifted to the left. Also, % inhibitions of PGI_2 (2.5 nM) and papaverine (0.1 mM) on aggregation were 2.6±1.1 and 3.1±0.9%, respectively. The % inhibition of the combined effect of PGI_2 and papaverine was 17.7±3.8%.

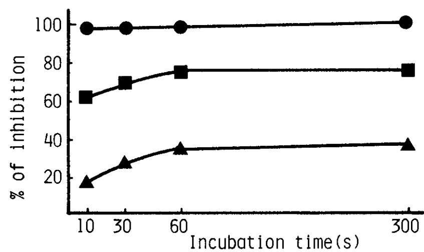

Fig. 1. Changes of Inhibitory Effect of Protopine during the Incubation of Platelet-Rich Plasma with Protopine on ADP-Induced Platelet Aggregation

—▲—; protopine 0.1 mM, —■—; protopine 0.25 mM, —●—; protopine 1.0 mM. Platelet-rich plasma was incubated with protopine at 37 °C for an appropriate length of time and ADP (50 μM) was added to the mixture solution. Each point represents the mean of 5 experiments.

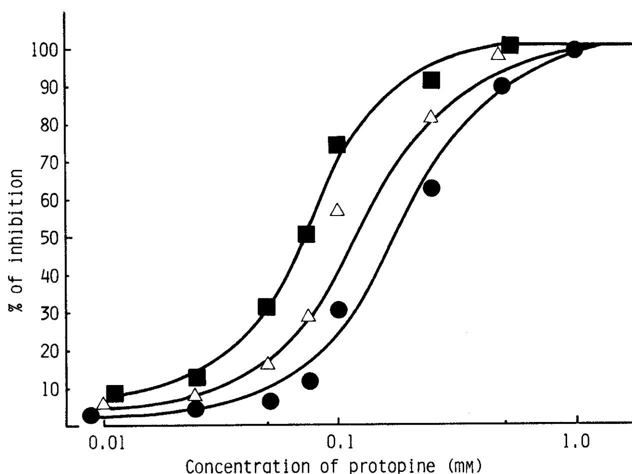

Fig. 2. Combined Effects of Protopine and PGI_2 or Papaverine on ADP-Induced Platelet Aggregation

—●—; protopine, —△—; protopine+PGI_2 2.5 nM, —■—; protopine+papaverine 0.1 mM. Platelet-rich plasma was incubated with either protopine and 2.5 nM PGI_2 or 0.1 mM papaverine at 37 °C for 3 min and ADP (50 μM) was added to the mixture solution. The % inhibitions of PGI_2 2.5 nM and papaverine 0.1 mM on ADP-induced platelet aggregation were 2.6±1.1 and 3.1±0.9%, respectively. The % inhibition of combined effect of PGI_2 and papaverine was 17.7±3.8%. Each point represents the mean of 5 experiments.

Cyclic AMP Content in Platelets As shown in Table I, the incubation of protopine at 0.25 or 1.0 mM with intact WPS significantly produced an increasing effect on cyclic AMP content in platelets. PGI_2 (1.0 or 2.5 nM) and papaverine (0.5 mM) also showed a significant effect.

The time course of cyclic AMP accumulation in papaverine (0.5 mM)-treated WPS in response to protopine (0.1 or 1.0 mM) or PGI_2 (10 nM) is shown in Fig. 3. These maximum stimulations of cyclic AMP content caused by protopine and PGI_2 occur after 60 s.

Adenylate Cyclase Activity As shown in Fig. 4, protopine produced a concentration–dependent elevation in the adenylate cyclase activity over basal activity (22.26±0.65 pM/mg protein/min). PGI_2 at 0.1, 1.0 nM also increas-

Table I. Effects of Protopine, PGI_2 and Papaverine on Platelet Cyclic AMP Content

Treatment	Concentration	Cyclic AMP content (pM/10^9 platelets)
Control		7.9±0.7
Protopine	0.01 mM	8.3±1.9
	0.05 mM	8.7±1.3
	0.25 mM	12.7±1.3[a)]
	1.0 mM	15.7±2.2[a)]
PGI_2	1.0 nM	15.1±2.2[a)]
	2.5 nM	42.2±2.6[b)]
Papaverine	0.5 mM	35.1±3.3[b)]

Washed platelet suspension (5.0×10^8 platelets/ml) was incubated with protopine, PGI_2 or papaverine at 37 °C for 1 min and 30% TCA was added to the mixture solution. The platelet cyclic AMP content was measured by radioimmunoassay. Each value represents the mean±S.E. of 3 experiments. Significantly different from control, *a)* $p<0.05$, *b)* $p<0.01$.

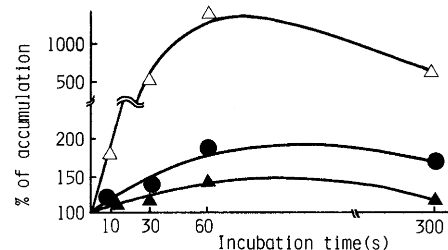

Fig. 3. Effects of Protopine and PGI_2 on Cyclic AMP Accumulation in Platelets

—▲—; protopine 0.1 mM, —●—; protopine 1.0 mM, —△—; PGI_2 10 nM. Washed platelet suspension (5×10^8 platelets/ml) was preincubated with 0.5 mM papaverine for 2 min at 37 °C. Then, either 0.1, 1.0 mM protopine or 10.0 nM PGI_2 was added and allowed to incubate for an appropriate length of time. The reaction was terminated by addition of 30% TCA and rapid freezing. The platelet cyclic AMP content was measured by radioimmunoassay. Each point represents the mean of 3 experiments.

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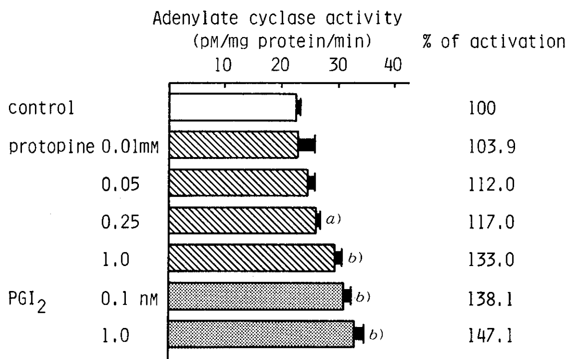

Fig. 4. Effects of Protopine and PGI_2 on Adenylate Cyclase Activity of Platelet Membrane Fraction

Platelet membrane fraction prepared from rabbit platelet was incubated with protopine or PGI_2 at 30 °C for 5 min and substrate solution containing 0.4 mM ATP was added to the mixture solution. After 20 min, the reaction was stopped by heating in a boiling water bath for 2 min. The cyclic AMP formed enzymatically from ATP was measured by radioimmunoassay. Each value represents the mean ± S.E. of 3 experiments. Significantly different from control, *a*) $p<0.05$, *b*) $p<0.01$.

ed the activity.

Cyclic AMP Phosphodiesterase Activity Cyclic AMP phosphodiesterase activity in platelets was decreased 54.3% by papaverine (0.25 mM) treatment. However, the cyclic AMP phosphodiesterase activity was not affected by protopine (0.01—1.0 mM) treatment (data not shown).

Discussion

It has been stated that there is a relation between the degree of inhibition of platelet aggregation and the increased intracellular cyclic AMP in platelets.[9] It is also known that PGI_2,[10] prostaglandin E_1[11] and ticlopidine[12] activate adenylate cyclase, while papaverine[13] and dipyridamole[13,14] promote accumulation of intracellular cyclic AMP through inactivation of phosphodiesterase.

In this work, the mode of action of protopine on the inhibition of platelet aggregation was investigated.

Protopine exhibited 50% inhibitory activity in a concentration of 0.15 mM on ADP-induced aggregation. Addition of a small amount of PGI_2 or papaverine, which is ineffective on ADP-induced aggregation, increased the inhibitory activity of protopine.

Protopine increased cyclic AMP amount in washed platelets and further increased the amount in papaverine-treated washed platelets. It also enhanced activity of crude adenylate cyclase prepared from washed platelets, whereas it showed no inhibitory activity on cyclic AMP phosphodiesterase.

These results suggest that protopine activates adenylate cyclase in the conversion of ATP to cyclic AMP. However, the concentration of protopine required to activate crude adenylate cyclase by 50% was over 1.0 mM, though protopine inhibited ADP-induced aggregation by 50% at a concentration of 0.15 mM. It was, therefore, suggested that the inhibitory activity of protopine on platelet aggregation can be attributed not only to the activation of adenylate cyclase but also involves some other mode of action.

Further studies are in progress on the effects of protopine on arachidonic acid and calcium metabolism in platelets.

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