Correlation between the Inhibition of Phosphorylation in Platelet Myosin Light Chains and the Inhibition of Platelet Aggregation by a New Calcium and Calmodulin Antagonist

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The recently synthesized compound [1,2,5-trimethyl-4-phenyl-4-β-(N-methyl-N-4'-methoxybenzyl)-ethylamino]piperidine dihydrochloride (AR-3), which is a derivative of [1,2,5-trimethyl-4-phenyl-4-β-(N,N-disubstituted-ethylamino)]piperidines, was tested for its effects on platelet aggregation and phosphorylation of light myosin chains isolated from platelets. AR-3 caused 50% inhibition of platelet aggregation in concentrations of 25.5 to 32.2 μM (depending on the aggregation inducer used) and 50% inhibition of light myosin chain phosphorylation in a concentration of 70 μM or, when 1 μM calmodulin was added, 120 μM. The good correlation found between the inhibitory effects of AR-3 on platelet aggregation and the phosphorylation of light myosin chains from platelets indicates that this compound inhibits platelet aggregation largely by inhibiting the Ca^{2+}-calmodulin-dependent phosphorylation of platelet myosin light chains, acting in this respect similarly to the well-known calmodulin antagonist W-7.

Key Words: *platelet aggregation; phosphorylation of myosin light chains; calmodulin antagonists*

Many years of intensive research efforts have resulted in unveiling of the highly complex morphofunctional structure of platelets, identification of their multiple physiological roles, and demonstration of their involvement in the pathogenesis of many diseases, including cardiovascular and neoplastic diseases [1,7]. In fact, a new dimension has been added to the prevention and pharmacotherapy of a number of diseases, based on regulation of the manifold functions performed by these cells. It has seen shown in numerous studies that platelets contain contractile proteins whose regulation is very similar to the regulation of contractile proteins in smooth muscle [2,5,6,9]. The main regulatory role in actin-myosin interactions in platelets is played by the myosin light chain of molecular weight 20,000 D [4]. Phosphorylation of the light chains is effected by the myosin light chain kinase which is activated by the Ca^{2+}-calmodulin (CM) complex. A good correlation was found between the Ca^{2+}-CM-dependent light-chain phosphorylation and the degree of platelet aggregation, as well as a correlation between the affinity of CM antagonists to CM and platelet aggregation [8].

In a large group of new compounds derived from [1,2,5-trimethyl-4-phenyl-4-β-(N,N-disubstituted-ethylamino)]piperidines, we identified compounds that can block Ca^{2+} entry into the cells and antagonize CM. The pharmacokinetics of these compounds has been investigated using many biochemical, physiological, and pathophysiological methods.

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In the present study, we tested one derivative from this series, [1,2,5-trimethyl-4-phenyl-4-β-(N-methyl-N-4'-methoxybenzyl)-ethylamino]piperidine dihydrochloride (designated AR-3), for its effects on platelet aggregation and light myosin chain phosphorylation in actomyosin complexes isolated from platelets. For comparison, compound W-7, a well-known CM antagonist, and the calcium-channel blocker verapamil were used.

MATERIALS AND METHODS

All isolation and purification procedures required to prepare platelet myosin were carried out at 4°C. Platelet actomyosin was extracted by Adelstein and Eisenberg's method [2] with some modifications. Packed platelets were obtained by sedimentation of platelet-rich plasma at 2000 g for 10 min, and packed platelets (5-10 g) were placed in 2 volumes of a solution containing 150 mM NaCl, 3 mM sodium citrate (pH 6.8), and 2.5 mM dithiothreitol and resedimented. This procedure was repeated several times. The purified platelets were suspended in 2 or 3 volumes of a solution containing 20 mM Tris-HCl (pH 7.5), 0.5 mM KCl, 2.5 mM dithiothreitol, 5 mM EDTA, 1 mM EGTA, 0.4 mM PMSF, and 0.1 mg/ml of soybean trypsin inhibitor, where they were subjected to lysis by adjusting the solution to 3% with respect of n-butanol. Thereafter the cells were extracted for 45 min and centrifuged at 78,000 g for 30 min. The supernatant was dialyzed for 24 h against 20 volumes of a solution containing 20 mM Tris-HCl (pH 7.5), 2.5 mM dithiothreitol, 5 mM EDTA, and 0.4 mM PMSF, after which the actomyosin precipitate was sedimented by centrifugation at 10,000 g for 20 min. The supernatant was discarded, while the actomyosin pellet was dissolved in 20 mM Tris-HCl (pH 7.5), 0.5 mM KCl, and 2.5 mM dithiothreitol and served as the source of platelet actomyosin.

Phosphorylation of light myosin chains in the platelet actomyosin samples was analyzed as follows:

TABLE 1. Effects of Compounds AR-3 and W-7 on Platelet Aggregation Induced by ADP, Collagen, Epinephrine, or Thrombin

Aggregation inducer	Compound, μM	
	AR-3	W-7
Collagen, 2 μg/ml	25.5±3.4	25.1±4.2
Thrombin, 0.125 U/ml	52.2±9.3	51.1±6.2
Epinephrine, 1 μg/ml	29.2±4.0	30.0±7.4
ADP, 5 μM	32.2±4.5	33.3±5.1

Note. The AR-3 and W-7 concentrations shown are those causing 50% inhibition of platelet aggregation.

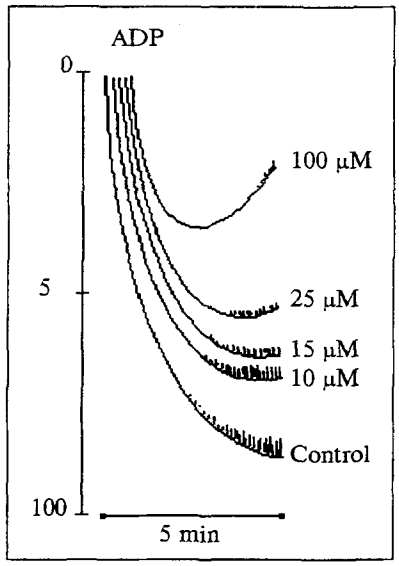

Fig. 1. Effect of AR-3 in different concentrations on ADP-induced (5 μM) platelet aggregation.

actomyosin was added in a concentration of 15 mg/ml was to a solution containing 20 mM Tris-HCl (pH 7.5), 125 mM KCl, 10 mM $MgCl_2$, and 100 mM $CaCl_2$ in the test samples and 5 mM EGTA in the control samples. The reaction was started by adding ATP at 25°C and arrested in 10 min by adding 1 ml of cold 10% trichloroacetic acid. After centrifugation of the reaction products at 3000*g* for 10 min, the pellet was dissolved in a solution containing 8 M urea, 30 mM Tris-glycine (pH 8.6), 0.2 mM EDTA, and 0.1% mercaptoethanol. The solution was homogenized and then centrifuged at 10,000*g* for 30 sec. This final supernatant containing 60 mg of protein was applied to gel. The degree of phosphorylation was estimated on a glycerol acrylamide gel as described [10]. The separating gel contained 10% acrylamide, 0.27% bis-acrylamide, 40% glycerol, and 0.375 M Tris-HCl (pH 6.8). The forming gel contained 3% acrylamide, 0.15% bis-acrylamide, 40% glycerol, and 0.12 M Tris-HCl (pH 6.8). Applied to the gel were samples containing 60 mg protein and 8 M urea. After electrophoresis, the proteins were stained with a 0.25% Coomassie blue (R-250) solution and then scanned in an GS 300 densitometer (Hoefer Scientific Instruments). The degree of phosphorylation was expressed as the percentage of the sum of both phosphorylated and nonphosphorylated electrophoretic peaks of the light chain (LC_2).

Platelet aggregation was examined by Born's method [3] using blood samples taken from fasting healthy subjects and mixed with an anticoagulant (3.8% solution of sodium citrate). Platelet-rich plasma was obtained by centrifugation of stabilized blood at 200 g for 10 min. Platelet aggregation was estimated from changes in the optical density of

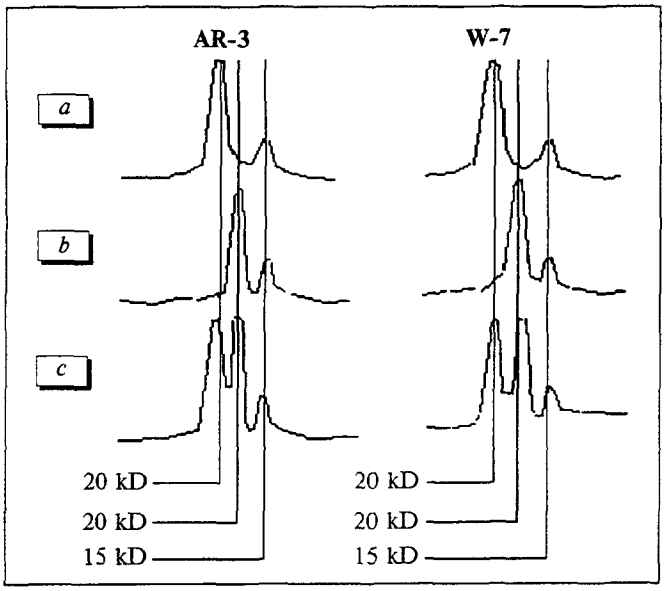

Fig. 2. Effects of compounds AR-3 and W-7 on phosphorylation of light myosin chains. *a*) dephosphorylated light chains; *b*) phosphorylated light chains; *c*) effect on light chain phosphorylation.

plasma recorded with a two-channel 300 BD-5 aggregometer.

RESULTS

In this study we examined the effect of the new compound AR-3 on platelet aggregation caused by different aggregation inducers and also considered the mechanism of its action. For comparison, the CM antagonist W-7 and the calcium-channel blocker verapamil were used.

AR-3 and W-7 both caused a dose-dependent inhibition of the platelet aggregation under the action of different inducers, the inhibitory effects being more marked when the incubation time was prolonged. Table 1 shows AR-3 and W-7 concentrations causing 50% inhibition of platelet aggregation induced by collagen, thrombin, epinephrine, and ATP at the indicated concentrations. The effects of different AR-3 concentrations on ADP-induced platelet aggregation are depicted in Fig. 1. Verapamil in this concentration range (10-100 μM) had no effect on the platelet aggregation induced by ADP, collagen, or thrombin, but decreased epinephrine-induced aggregation (data not shown), which is explained by the direct verapamil action on α_2-receptors.

Tests of AR-3 and W-7 for their effects on the phosphorylation of myosin LC_2 showed that these compounds also inhibit the Ca^{2+}-CM-dependent LC_2 phosphorylation in a dose-dependent manner in the concentration range of 10-150 μM, 50% inhibition being observed at 70 μM with AR-3 and 80 μM with W-7 (Fig. 2). Higher AR-3 and W-7 concentrations (120 and 130 μM, respectively) were, however, required to achieve 50% inhibition of LC_2 phosphorylation when CM was added (1 μM). Verapamil had no effect of LC_2 phosphorylation in the concentration range of 10 to 200 μM.

The results of this study indicate that the compound AR-3, like W-7, inhibits platelet aggregation largely by inhibiting the Ca^{2+}-CM-dependent phosphorylation of platelet myosin LC_2.

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