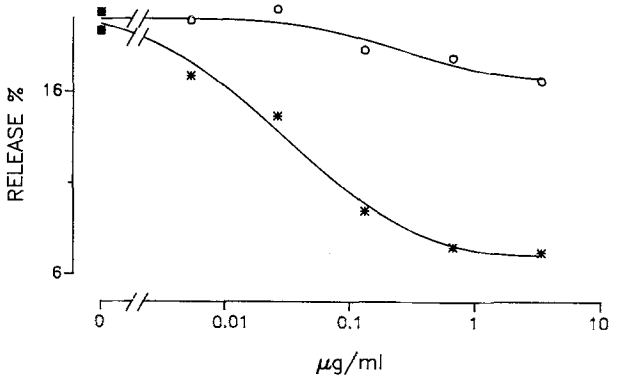

Figure 3. Inhibition of the K^+-evoked [^{3}H] GABA release by tetanus as compared with botulinum A toxin. Ordinate and abscissa as in figure 2C. (■) Toxin-free controls.

2% w/v) under otherwise identical conditions. Tetanus toxin and botulinum A toxin (both 1.67 μg/ml) reduced the fractional evoked release from 25.5 to 11 and 4.5%, respectively. Ten-fold lower concentrations of tetanus toxin were no longer active (22.5%), whereas botulinum A toxin still was (15.5%, all values are the means of duplicates). Throughout their work Heredero and Oja [1] used one single tetanus toxin concentration (1.5×10^3 MLD/ml). This concentration (about 75 ng/ml as calculated from an LD_{50} of 2 ng/kg) was too low to have an effect on our slices, too, but was clearly effective (see fig. 2) on brain homogenate. The finding demonstrates again a disadvantage of slices for studying tetanus toxin action on transmitter release.

Tetanus toxin also inhibits the release of [^{3}H]GABA (fig. 3). In contrast to noradrenaline release, botulinum toxin was slightly active, but one to two orders of magnitude less potent than tetanus toxin. Moreover, neither toxin led to an accumulation of [^{3}H]GABA (not shown), in contrast to [^{3}H]noradrenaline (see figs 1 and 2).

The data corroborate our previous finding [2] that tetanus and botulinum A toxin inhibit both noradrenaline and GABA release. Although botulinum A toxin can ascend to the spinal cord [6], its very low potency as an inhibitor of GABA release explains why this toxin does not share the tetanic action in vivo. The slightly higher inhibitory potency of botulinum A as compared with tetanus toxin on noradrenaline release fits in with our recent observation on primary nerve cell cultures from rat brain cortex. Here botulinum A toxin was also more potent than tetanus toxin [5]. In our previous experiments [2] botulinum A toxin had been less potent than tetanus toxin in inhibiting noradrenaline release. The difference may be due to the superior purity and preservation of the presently available botulinum A neurotoxin.

Currently we are using inhibition of noradrenaline release from superfused brain homogenate to measure the in vitro activity of all clostridial neurotoxins or their fragments. The system also furnishes functional in vitro data for the titration of antibodies.

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Effect of AD6 (8-monochloro-3-beta-diethylamino-ethyl-4-methyl-7-ethoxycarbonylmethoxy coumarin) on cyclic nucleotide phosphodiesterases in human platelets

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Summary. The effect of AD6 (8-monochloro-3-beta-diethylamino-ethyl-4-methyl-7-ethoxycarbonylmethoxy coumarin), an inhibitor of platelet aggregation, on cyclic nucleotide metabolism was investigated. AD6 inhibited selectively human platelet cyclic GMP phosphodiesterase, which was separated from cyclic AMP phosphodiesterase by DEAE-cellulose chromatography. Addition of AD6 to washed platelets increased cyclic GMP levels significantly in two minutes. These results could be useful in elucidating the action of AD6 on intact platelets.
Key words. AD6; cyclic GMP; cyclic nucleotide phosphodiesterase inhibition; platelets.

Cyclic AMP is clearly implicated in platelet function, acting as a negative modulator and possibly affecting the intracellular calcium homeostasis [1]. An increase in cyclic AMP levels causes an inhibition of platelet aggregation, whereas agents reducing cyclic AMP levels lead to or potentiate platelet aggregation. On the other hand, the role of cyclic GMP remains controversial, since both aggregating and disaggregating effects have been described [2]. Although cyclic GMP has been reported to increase in human platelets stimulated by thrombin or collagen [1], it has been proposed recently that cyclic GMP could exert inhibitory effects on platelet activation [3]. This effect has been related to calcium mobilization, both through the plasma membrane and from internal stores [4].

Cyclic nucleotides may be elevated intracellularly in a selective manner, through the action of phosphodiesterase (PDE) inhibitors [5]. Some inhibitors, like RO 15-2041, do not increase cyclic nucleotide levels at a concentration which affect

platelet function, but in this case a separate regulation of degradation of cyclic AMP in different compartments has been suggested[6].

AD6 is a coumarine derivative with several cardiovascular activities. It has been shown to be a powerful inhibitor of platelet activation induced by a variety of stimuli including epinephrine, PAF, ADP, A23187 and thrombin, but its mechanism of action is poorly understood[7,8]. In order to clarify this mechanism better, we studied the effect of AD6 on different forms of PDE from human platelets and on the cyclic nucleotide content of the platelets.

Materials and methods. Cyclic AMP, cyclic GMP and *Ophiophagus hannah* snake venom were purchased from Sigma (St. Louis, MO). AD6 was kindly given by Fidia Neurobiological Research Laboratories (Abano Terme, Italy). Anion exchange resin (AG1-X8) was purchased from Bio-Rad. Cyclic [^{3}H]AMP and cyclic [^{3}H]GMP were purchased from Amersham. All other chemicals were reagent grade.

Platelet PDE preparation. Outdated platelet concentrates were obtained from Centro Trasfusionale, Ospedale S. Orsola, Bologna. The suspensions were made 1 mM in EDTA and centrifuged for 15 min at 300 × g to remove most of the contaminating erythrocytes. The platelets were then pelleted by centrifugation at 1200 × g, washed in 100 mM Tris-acetate, pH 6.0 containing 1 mM EDTA and 0.15 M NaCl and stored at − 80 °C.

Phosphodiesterase was prepared as described previously[9,10]. The frozen platelets were resuspended in 50 mM Tris-acetate, pH 6.0 containing 1 mM $MgCl_2$, 2 mM EGTA, 1 mM phenylmethanesulfonyl fluoride and 3 mM 2-mercaptoethanol (buffer A) and homogenized using an Ultra-Turrax homogenizer for 2 min. The homogenized solutions were sonicated (30 s/ml) and the soluble PDE preparation was obtained by centrifugation at 100,000 × g for 90 min. The supernatant was applied to a DEAE-Sephacel (Pharmacia, Uppsala, Sweden) column (1.6 × 20 cm) equilibrated with buffer A and the column was washed with several bed volumes of the same buffer at a flow rate of 30 ml/h. A linear gradient from 0 to 1 M sodium acetate in buffer A (total volume of 300 ml) was then applied. The column fractions were assayed at 1.25 and 125 µM cyclic AMP and cyclic GMP.

PDE assay. Phosphodiesterase activity was measured by the method of Thompson et al.[11]. The reaction mixture (0.4 ml) contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, cyclic [^{3}H]AMP or cyclic [^{3}H]GMP, an appropriate amount of purified enzyme and various concentrations of AD6. After 20 min incubation at 30 °C, the reaction was stopped by boiling for 1 min, 100 µg of snake venom were added and the mixture was incubated for 10 more min. Then 1 ml of anion-exchange resin in 20% methanol was added, and after centrifugation an aliquot of the supernatant was counted in a liquid scintillation counter.

Measurement of drug-treated platelet cyclic GMP. Blood obtained from healthy volunteers was rapidly citrated. Platelet-rich plasma (PRP) was prepared by centrifugation at 2000 × g for 90 s. A washed platelet suspension was prepared as described by Kinlough-Rathbone et al.[12]; the only modification was that prostacyclin was added to the first and second washing fluids. Platelets were finally suspended in Tyrode buffer containing 3.5 mg/ml of albumin, at a final concentration between 2.5 and 3.5×10^{11}/l.

One-ml aliquots of washed platelets were incubated at 37 °C for 2 min with various concentrations of drugs. The reaction was stopped by boiling for 1 min. The mixtures were sonicated and centrifuged at 10,000 × g. Cyclic GMP was measured by radioimmunoassay using the Amersham cyclic GMP assay kit.

Results. Inhibition of platelet cyclic nucleotide phosphodiesterases by AD6. The effect of AD6 on human platelet cyclic nucleotide phosphodiesterases was examined on a cyclic GMP-specific PDE (K_m = 1 µM) and on a cyclic AMP-specific PDE resolved using DEAE-cellulose chromatography. Calmodulin did not stimulate the activity of either form; this was in agreement with Weishaar et al.[13].

The concentrations of AD6 producing 50% inhibition of cyclic GMP PDE and cyclic AMP PDE were about 200 µM and greater than 1.2 mM respectively, as the substrate concentrations were 1 µM. The inhibition of cyclic GMP PDE by AD6 was further analyzed by means of a Dixon plot (fig. 1), giving a K_i value of 200 µM.

Since calcium-calmodulin antagonists like W-7 are potent inhibitors of platelet aggregation[5], the effect of AD6 has been also investigated on a calcium-calmodulin sensitive PDE partially purified from bovine brain; the drug had a poor inhibitory effect on the stimulated enzyme (data not shown).

Effect of AD6 on cyclic nucleotide levels. AD6 (0.1 mM) has no effect on cyclic AMP levels in washed human platelets[7]. On the other hand, as shown in figure 2, AD6 significantly elevated cyclic GMP levels, and the level was increased about 2.5 times at a drug concentration of 0.1 mM. The maximal response is reached within 2 min. The action of AD6 has been compared with that of dipyridamole, which inhibits selectively cyclic GMP phosphodiesterase. Dipyridamole (0.1 mM) increased the cyclic GMP content in platelets 3.3-fold, in agreement with Hagiwara et al.[14].

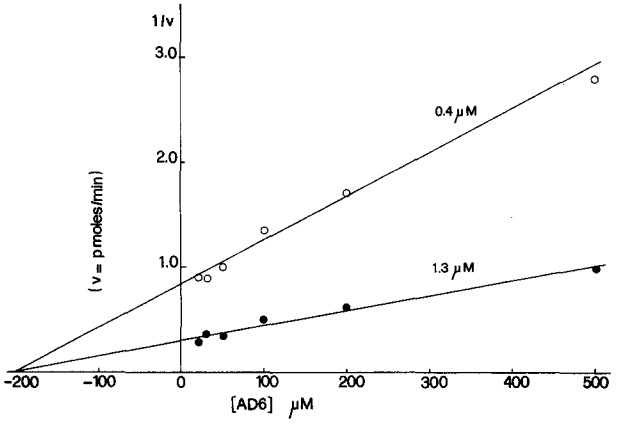

Figure 1. Dixon plot of inhibition of cyclic GMP phosphodiesterase by AD6. Substrate concentrations were 0.4 µM and 1.3 µM.

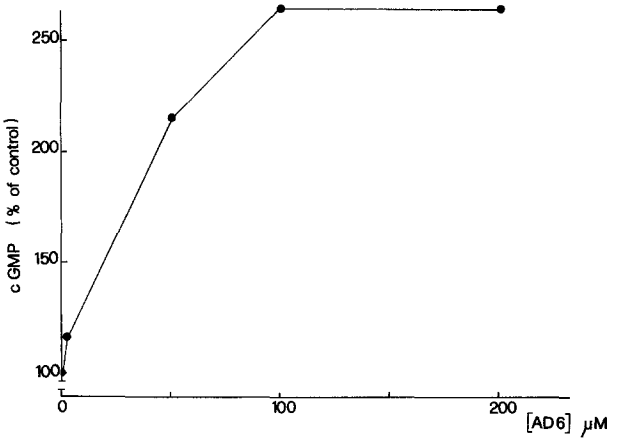

Figure 2. Effect of AD6 on cyclic GMP level. Washed human platelets were incubated for 2 min with various concentrations of AD6. Each point is the mean of at least three different samples from separate experiments.

Discussion. Cyclic GMP has been shown to be increased by aggregatory agents such as thrombin and collagen, but this elevation may represent a feedback control of aggregation. Indeed, several agents that stimulate cyclic GMP formation inhibit platelet reactivity. Smooth muscle relaxants like sodium nitroprusside and nitric oxide have been reported to elevate platelet cyclic GMP levels, effectively preventing or reversing platelet aggregation[2]. The mechanism underlying this effect is still under discussion.

AD6 is an experimental drug with various activities on the cardiovascular system and inhibits, dose-dependently, platelet aggregation induced by several stimuli[7,8]. Among the mechanisms involved in platelet aggregation, an AD6 action on cyclic AMP levels through a cyclic AMP PDE inhibition is unlikely. In fact, Prosdocimi et al. showed that the drug did not modify cyclic AMP accumulation induced by prostacyclin[7]. On the other hand, the present study indicates that AD6 elevates the cyclic GMP level significantly in washed human platelets. We have obtained a similar increase after treatment with dipyridamole. These findings were supported by results from enzymatic analyses which showed that AD6 selectively inhibited cyclic GMP PDE. Thus, AD6-induced elevation of platelet cyclic GMP seems to be related to the inhibition of cyclic GMP hydrolysis, although other effects cannot be ruled out. It has been proposed recently that cyclic GMP may prevent phospholipase C activation, resulting in a reduced formation of IP_3 and in a suppression of calcium mobilization[4].

It has been suggested that antiaggregating properties of AD6 are likely to be related to an inhibition of the release of arachidonic acid[15]. It is interesting to compare the action of AD6 to mepacrine, a phospholipase A_2 inhibitor. This drug blocks arachidonate release in platelets, increasing at the same time the level of cyclic GMP, and it has been proposed that the increase in the cyclic GMP level may be partially related to the mepacrine-induced change in arachidonate metabolism[16–18].

Our findings that AD6 inhibits cyclic GMP phosphodiesterase and elevates platelet cyclic GMP levels could be important in understanding the action of AD6 in intact platelets.

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Enhancement of the cytogenetic efficacy of the antitumor agent bleomycin by the calcium and calmodulin antagonist fendiline

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Summary. The induction of chromosome aberrations (dicentric and ring chromosomes) in human lymphocytes by the antitumor agent bleomycin is synergistically enhanced when bleomycin is applied together with the calcium antagonist fendiline (Sensit®).
Key words. Bleomycin; calcium antagonist; calmodulin antagonist; chromosome aberrations; comutagenesis; cytostatics; fendiline; lymphocytes; verapamil.

Recently we have shown that the calcium antagonist verapamil (Isoptin®) enhances synergistically the cytogenetic efficacy of the antitumor agents bleomycin and peplomycin[1]. In these in vitro studies the yield of chromosome aberrations (dicentric and ring chromosomes) induced in human lymphocytes was drastically increased when verapamil was applied together with the antitumor agent. Verapamil alone exhibited no mutagenic effect. Besides this demonstration that verapamil is a comutagen our results appear to be interesting for two other reasons: not only the *cytogenetic* efficacy of bleomycin and peplomycin is enhanced synergistically by verapamil[1], but also the *cytotoxic* efficacy (cell killing activity) of various antitumor agents including bleomycin[2–6]. Furthermore, mitotic index reduction induced by bleomycin and peplomycin is synergistically increased by verapamil[7].

We were interested to see whether the cytogenetic efficacy of bleomycin is also potentiated by another calcium antagonist, fendiline (Sensit®). This substance is an aliphatic amine, namely N-(3,3-diphenylpropyl)-α-methylbenzyl-aminehydrochloride and exhibits besides calcium-antagonistic also calmodulin-antagonistic properties[8].