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Anagrelide: a potent and selective inhibitor of platelet cyclic AMP phosphodiesterase enzyme activity

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Anagrelide has been characterized previously as a broad spectrum inhibitor of platelet function in a variety of models [1, 2]. It inhibits the *in vitro* aggregation of rabbit and human platelets induced by all the agents that have been tested (ADP, collagen, thrombin, platelet activating factor, antigen/antibody complexes, and arachidonic acid) and also inhibits *ex vivo* aggregation in a variety of species including humans. When *in vivo* models are considered, anagrelide is an effective inhibitor of biolaser-induced intravascular thrombosis in rabbit ears and of electrically-induced carotid artery thrombosis in dogs [2]. The cardiovascular effects of anagrelide in anesthetized dogs include a modest decrease in mean aortic blood pressure probably due to vasodilation [2, 3] and a positive inotropic effect that is particularly prominent in dogs in which cardiac function has been compromised by propanolol [3].

The biochemical mechanism(s) for these effects of anagrelide has not been investigated in any detail. In the only biochemical study published to date, Tang and Frojmovic [4] examined the effects of anagrelide on cyclic AMP metabolism in human platelets. They found that the compound inhibits cyclic AMP phosphodiesterase (PDE) activity by approximately 70% at concentrations of 10 and 50 μM when the substrate concentration is low (0.83 and 0.03 μM), whereas inhibition is less than 25% at higher substrate concentrations. In the same studies, an increase in platelet cyclic AMP levels was not demonstrated although synergism between anagrelide and prostaglandin E_1 with respect to inhibition of platelet aggregation was found. On the basis of these data, Tang and Frojmovic concluded that anagrelide was probably an inhibitor of a "low K_M" cyclic AMP PDE within the human platelet. The present study was undertaken to explore further the effects of anagrelide on cyclic nucleotide PDE enzyme activities in platelets and in other tissues. The compound has been found to preferentially inhibit the cyclic AMP PDE activity in platelets. Several other PDE inhibitors including milrinone, 3-isobutyl-1-methyl-xanthine (IBMX) and theophylline have also been studied for purposes of comparison with anagrelide.

Materials and methods

Preparation of platelet sonicates, tissue supernatant fractions and compounds. Platelets were prepared from freshly drawn blood from human donors who had given informed consent and from rabbits. The citrated blood was centrifuged at 120 *g* for 15 min at 4°, and the platelets were subsequently pelleted from the supernatant fraction by centrifugation at 1000 *g* for 15 min. The platelet pellets were frozen and thawed three times, then resuspended in 0.25 M sucrose, and sonicated (3 × 30 sec, 60 W, Bronwell Biosonic III sonicator). With human donors, platelets from

90 ml of blood (30 ml from three donors) were prepared in 6 ml of sucrose solution. PDE activity was assayed using a 1:120 dilution of this sonicated platelet preparation.

Tissues taken from animals being used for other purposes were homogenized in 0.25 M sucrose (1:5 or 1:10 w/v) using a Brinkmann polytron homogenizer. The homogenates were centrifuged at 30,000 *g* for 20 min, and the supernatant fractions were stored at −20°. The dilutions of the supernatant fractions used for the PDE assay ranged from 1:400 to 1:8000 (w/v). Each dilution was chosen such that enzyme activity was linear over a 10-min time interval and was proportional to tissue concentration. Anagrelide and milrinone were dissolved in dimethyl sulfoxide (DMSO) at 10^{-2} M and diluted in buffer. The relevant concentration of DMSO did not affect the PDE assay.

PDE assay and purification. PDE enzyme activity was measured as described by Thompson *et al.* [5]. Diluted tissue supernatant or platelet sonicate was preincubated with compounds in duplicate at 30° for 5 min in a buffer consisting of 40 mM Tris–HCl, 5 mM $MgCl_2$ and 3.75 mM mercaptoethanol, pH 8.0. [^{3}H]Cyclic AMP or [^{3}H]cyclic GMP (0.125 or 0.25 μM and approximately 440,000 dpm) was added and the tubes were incubated for 10 min, after which they were boiled for 2 min, placed on ice and snake venom (Sigma Chemical Co.) added to each tube. After a second 10-min incubation at 30°, 1 ml of a 1:2 (v/v) slurry of ion exchange resin (AG1-X2 200–400 mesh, BioRad Laboratories) was added. The tubes were centrifuged, and a portion of the clear supernatant was counted in a liquid scintillation counter. The difference between duplicate measurements rarely exceeded 10%.

The various PDE enzyme activities in a 100,000 *g* supernatant fraction prepared from rabbit heart were partially purified and separated from each other as described by Thompson *et al.* [5]. Rabbit heart was chosen for this study because it is a good source of PDE enzyme activity and also because heart tissue from several species has been used in studies of PDE enzymes by others. Briefly, supernatant was applied to DEAE cellulose columns and eluted with sodium acetate buffer, pH 6.5, which increased in molarity in a linear fashion from 70 mM to 1 M. The enzyme activity was stabilized by the addition to the collection tubes of ethylene glycol (30% final concentration), ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (1 mM), and phenylmethylsulfonyl fluoride, pepstatin, and leupeptin (10^{-4} M each). Fractions were stored at −20°.

Results and discussion

The effects of anagrelide and also of milrinone, theophylline and IBMX on cyclic AMP phosphodiesterase enzyme activity in platelet sonicate and in supernatant fractions from a variety of tissues are presented in Fig. 1. The effects of the compounds on human platelet cyclic GMP enzyme activity are also included. As can be seen, anagrelide was a potent inhibitor of the enzyme activity in the two platelet preparations with IC_{50} values of $5.4 \pm 1.4 \times 10^{-8}$ M (N = 9) and 4×10^{-8} M (N = 1) for the human platelet and rabbit platelet respectively. In contrast, anagrelide failed to inhibit enzyme activity in the other tissues by more than 50% even at 10^{-4} M. With the majority of these tissues, however, it can be seen (Fig. 1) that half-maximal inhibition occurred at a concentration below 10^{-7} M. Cyclic GMP inhibited the human platelet cyclic AMP PDE activity with an IC_{50} value of 4×10^{-7} M. When cyclic GMP PDE activity was considered (dashed line, Fig. 1), anagrelide inhibited the enzyme activity from human platelets but with a considerably lower potency than it inhibited the cyclic AMP enzyme activity. Cyclic GMP PDE was studied in many of the other tissues included in Fig. 1 (data not shown). In all cases, inhibition was considerably less than the inhibition of the cyclic AMP enzyme in the same tissue. As anticipated, IBMX and theophylline inhibited cyclic AMP PDE in all the tissues studied and also cyclic GMP in human platelets to very similar extents (Fig. 1) with IC_{50} values between 2 and 5×10^{-6} M for IBMX and between 1 and 3×10^{-4} M for theophylline. Milronone, a positive inotropic agent used in the treatment of congestive heart failure [6], has been described as an inhibitor of cardiac PDE enzyme activity

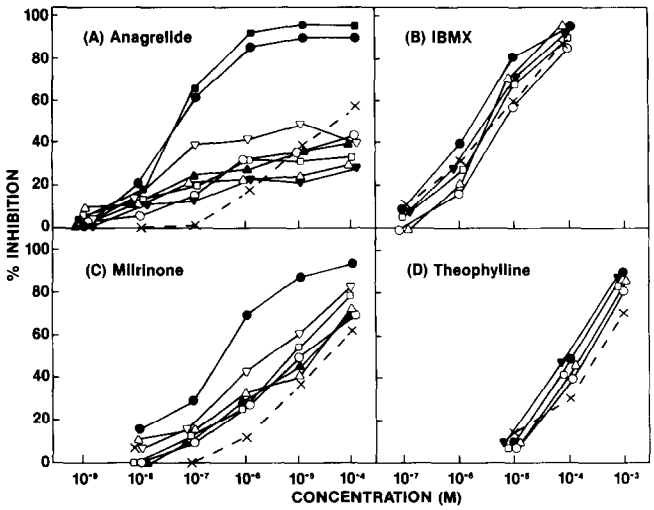

Fig. 1. Effects of anagrelide, IBMX, milrinone and theophylline on cyclic nucleotide PDE enzyme activity in various tissues. Cyclic AMP enzyme activity: (●) human platelets; (■) rabbit platelets; (▲) rabbit heart; (▼) guinea pig heart; (○) guinea pig liver; (□) guinea pig lung; (△) rabbit aorta; and (▽) dog femoral artery. Cyclic GMP enzyme activity: (×--×) human platelets. The platelet sonicate was prepared and diluted as described in Materials and methods. The 30,000 *g* tissue supernatant fractions were studied at dilutions (v/w) of 1:400 (dog femoral artery), 1:800 (guinea pig lung), 1:1200 (guinea pig liver, rabbit aorta), 1:4000 (rabbit heart) and 1:8000 (guinea pig heart). The number of replicates varied from 1 (anagrelide–rabbit platelets and heart, anagrelide and milronone–dog femoral artery) to 9 (anagrelide–human platelets) and was most frequently 2 or 3.

[7, 8] and, more recently, of platelet PDE activity [9]. As can be seen in Fig. 1, panel C, milrinone inhibited cyclic AMP PDE enzyme in human platelets in the present study and showed some specificity for this enzyme activity although its selectivity and potency were not as great as those of anagrelide. The IC_{50} noted in the present study (2×10^{-7} M) is close to that found by Macphee *et al.* [9] (4.6×10^{-7} M) using a substrate concentration of 0.35 μM cyclic AMP. Amrinone, a close relative of milrinone, was included in the present study. Its pattern of inhibition was closely similar to that seen with milrinone except that it was less potent throughout with an IC_{50} as an inhibitor of human platelet cyclic AMP PDE of 6×10^{-6} M compared with 2×10^{-7} M for milrinone.

In their recent paper describing the purification of human platelet PDE, Macphee and his colleagues described the proteolytic alteration of the purified platelet low K_M cyclic AMP PDE [9]. In the present study, protease inhibitors were not included in the platelet sonicate. It is very likely, therefore, that the proteolytically degraded forms of enzyme were routinely studied. In a special comparison, sonicates of human platelets were prepared with and without protease inhibitors (50 mM benzamidine, 20 μg leupeptin per ml), and the inhibition by anagrelide was determined. IC_{50} Values of 2×10^{-8} M (with protease inhibitors) and 3×10^{-8} M (without inhibitors) were noted. While protection of the PDE against degradation could not be verified in the impure sonicate control, enzyme activity was greater in the preparation that did not contain proteases consistent with the proteolytic activation of enzyme activity described by Macphee *et al.* [9].

The phosphodiesterase enzyme activity from human platelets has been determined by Grant and Colman [10] and also be Macphee *et al.* [9] to be largely a "low K_M" cyclic AMP enzyme that has been called PDE fraction III. In addition, milrinone has been characterized as a preferential inhibitor of this PDE [9, 11]. Because of these observations, anagrelide was studied as an inhibitor of PDE fractions I, II and III separated from each other from rabbit heart supernatant as described under Materials and methods. Anagrelide did not inhibit PDE I or II except at a concentration of 10^{-4} M where inhibition of 33 and 39%, respectively, was noted. As expected, anagrelide inhibited PDE fraction III with a dose–response curve that was closely similar to that seen in the human platelet preparation. The IC_{50} for inhibition of the rabbit heart PDE fraction III was 7×10^{-8} M. Cyclic GMP also inhibited this enzyme activity with an IC_{50} of 2×10^{-7} M. A Lineweaver–Burk plot of the PDE fraction III activity is shown in Fig. 2. The substrate dose–response curve was not linear at higher substrate concentrations. This kinetic pattern has been shown by others for low K_M cyclic AMP PDEs [12, 13] and attributed to cooperativity effects. Recent studies suggest it is more likely to be due to multiple proteolytic forms of the fraction III PDE [9] or contamination of the PDE preparation with an additional distinct PDE enzyme [14]. In any event, the K_M value found when lower substrate concentrations (0.125 to 0.5 mM) were analyzed (3×10^{-7} M, Fig. 2) is closely similar to the K_M value found by Grant and Colman [10] for human platelet PDE III (2×10^{-7} M). It can also be seen that both anagrelide and theophylline inhibited the rabbit heart PDE fraction III activity with kinetic characteristics that are compatible with a competitive mechanism. K_I values calculated from the linear portions of the curves were 2×10^{-8} M for anagrelide and 4×10^{-5} M for theophylline.

In summary, the data in the present paper provide support for the conclusion that anagrelide is a potent and selective inhibitor of PDE activity in human and rabbit platelets and also of a low K_M cyclic GMP inhibitible cyclic AMP PDE in rabbit heart. It is suggested that the partial inhibition noted by anagrelide of PDE activity in supernatant fraction from tissues other than platelets reflects the content of this PDE isozyme in these various preparations. The specificity demonstrated by anagrelide should make it a suitable tool for the *in vitro* study of PDE enzyme types. Hopefully, it will also prove to be useful in clinical situations where selective inhibition of platelet function is desirable.

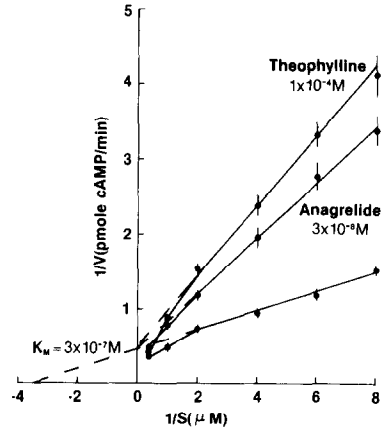

Fig. 2. Lineweaver–Burk plot of cAMP PDE III partially purified from rabbit heart 100,000 *g* supernatant fraction.

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