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INHIBITION OF HEART AND BRAIN CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE BY NEW NON-STEROIDIC COMPOUNDS STRUCTURALLY RELATED TO NATURAL CARDENOLIDES

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Abstract—AP 10 and related compounds are non-steroidic analogs of cardenolides which exhibit cardiotonic effects on mammals and amphibians isolated hearts. These synthetic compounds were effective inhibitors of the high affinity cyclic AMP phosphodiesterase of beef heart, rat heart and rat brain. AP 10 was the best inhibitor among them. It was approximately ten times as potent as theophylline and three times less effective than MIX and papaverine. Its affinity for the heart enzyme was eight to ten times higher than for the brain enzyme. The inhibition produced by AP 10 was competitive, reversible and was not reversed by high concentrations of magnesium ions. AP 10 slightly affected the binding of cyclic AMP with specific binding protein, but it had a far lower affinity for the binding sites than cyclic AMP. The possibility that inhibition of the low K_m phosphodiesterase by AP 10 and related compounds may contribute to their cardiotonic action is discussed.

There is a lot of evidence suggesting that cyclic adenosine 3′,5′-monophosphate (cyclic AMP) plays a key role as intracellular mediator for the actions of many hormones or neurotransmitters [1, 2].

The level of cyclic AMP in the cell is controlled, in part, by a group of cyclic AMP phosphodiesterases (EC 3.1.4.17) (PDEs), the only known enzymes able to hydrolyze cyclic AMP to 5′AMP. This conversion by phosphodiesterases is the major physiological pathway for the termination of the intracellular effects of cyclic AMP. Therefore, several investigators consider this enzyme system as a "specific target" for the development of new drugs [3–5].

Cyclic nucleotide phosphodiesterases are widely distributed in various tissues and organs, specially in the brain and in the heart (for review, see [1–5]). It is well documented now that cyclic AMP is involved in the control of cardiac contractility [6]. Inhibitors of phosphodiesterases are known to increase cardiac contractility and to potentiate the inotropic effects of adenylate cyclase stimulatory agents. Some of these drugs, such as theophylline, increase myocardial contractility by themselves. Recently, Holzmann *et al.* [7] reported that papaverine markedly increases the force of contraction in isolated guinea-pig auricles.

On the other hand, some cardioactive drugs are known to inhibit cyclic nucleotide phosphodiesterases. For example, Lippman showed that compound A.Y 17 605, isomeric to the natural cardiac glycosides, is a more potent inhibitor of PDEs than theophylline [8].

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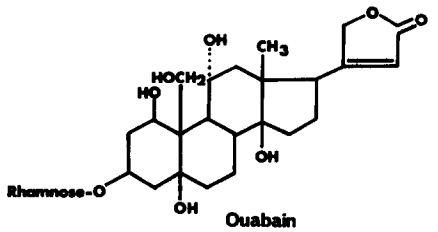
The compounds considered in the present studies (Fig. 1) are synthetic analogs of natural cardenolides. Their positive inotropic effects on mammals and amphibians isolated heart were reported previously [9, 10]. They have been shown to inhibit erythrocytes ATPases (EC 3.6.1.3), with the particularity for the compounds AP 10 and IP 17, to affect Mg^{2+}-dependent ATPases [9, 10]. In contrast, natural cardenolides as ouabain only affect Na^+, K^+-dependent ATPases.

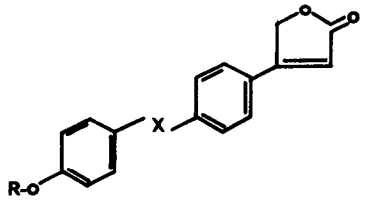
On account of the relation which likely exists between inotropic effects and inhibition of PDEs [6–8], it was of interest to study the effects of our cardenolides analogs on these enzymes. This study was carried out comparatively to well-known PDEs inhibitors such as theophylline, 3-isobutyl-1-methylxanthine (MIX) and papaverine.

Kinetic studies have shown that most tissues, including brain [11] and heart [12, 13] contain phosphodiesterases with a high and a low K_m for cyclic AMP. The low K_m is in the range of the intracellular concentration of cyclic AMP. This low K_m activity seems to be involved in the regulation of cyclic AMP level under normal physiological conditions [4, 5]. So, we investigated the inhibitory effects of our cardenolides analogs on this high affinity (low K_m) form of phosphodiesterase.

There appear to be both structural and kinetic differences between the phosphodiesterases isolated from different tissues. These differences are reflected in the differential sensitivities to drugs of the enzymes from different sources [4, 5, 14, 15]. So, in order to investigate an eventual tissue selectivity of our drugs, we studied their effects on brain and heart PDE preparations.

We present here the results obtained with PDEs from beef and rat heart and from rat brain.





AP 10 : R = Glucose
X = CO-CH2

IP 17 : R = CH2-COOH
X = CO-CH2

IP 24 : R = Glucose
X = CO-NH

Fig. 1. Structures of compounds studied.

MATERIALS AND METHODS

[8-^{3}H]adenosine 3',5'-monophosphate (ammonium salt, 26 Ci/m-mole) and [2-^{3}H]adenosine (24 Ci/m-mole) were supplied by the Radiochemical Centre, Amersham. Unlabeled cyclic AMP, 5'-nucleotidase from Snake (Ophiophagus hannah) venom, theophylline, papaverine and ouabain were purchased from Sigma Chemical Co. (St. Louis, MO). Anion-exchange resin, Bio-Rad AG 1-X2 (200–400 mesh) was obtained from Bio-Rad Labs; 3-isobutyl-1-methylxanthine (MIX) was from Aldrich Europe (Beerse, Belgium). The other reagents were of analytical grade.

Enzyme preparations. The beef heart PDE (Sigma Chemical Co.) was a lyophilized preparation. The rat brain PDE was prepared as described previously by Brooker *et al.* [16] with slight modifications. Adult male Sprague–Dawley rats (150–200 g) were killed by decapitation and the brains were rapidly removed. All following steps were performed at 4°. The cortex was homogenized in 10 vols (w/v) of distilled water, in a glass Potter homogenizer with a tight-fitting teflon pestle, and centrifuged at 48,000 *g* for 30 min. The supernatant was made 50 per cent saturating in ammonium sulfate. The pellet resulting from centrifugation for 25 min at 30,900 *g* was dissolved in a minimal volume of 60 mM Tris–HCl, 5 mM mercaptoethanol (pH 8.0) buffer, and dialyzed overnight against the same buffer with two changes. This preparation was used as source of PDE activity.

The rat heart PDE was prepared according to a modified version of that described by Butcher [17] for bovine heart PDE. Hearts from decapitated adult rats (Sprague–Dawley) were excised and immediately rinsed with cold saline solution (NaCl 9% w/v), then with 40 mM Tris–HCl (pH 7.4) buffer containing sucrose 0.33 M. The myocardial tissue was minced with scissors and homogenized at 4° for 20 sec in 4 vols (w/v) of the same buffer, using an Ultra-Turrax mixer at maximal speed. The crude extract was then treated as described above for the rat brain preparation.

Enzyme assay. Cyclic AMP phosphodiesterase activity was assayed by the two step radioisotopic procedure of Thompson and Appleman [11]. This method involves phosphodiesterase hydrolysis of [^{3}H]cyclic AMP to [^{3}H]5'-AMP, which is further converted to [^{3}H]adenosine by snake venom nucleotidase. Unreacted cyclic AMP is removed by binding to Bio-Rad AG 1 X2 resin. The supernatant solution containing [^{3}H]adenosine is then counted by liquid scintillation techniques [11].

By varying the concentrations of cyclic AMP between 0.125–125 μM, the high and low K_m for each PDE preparation were determined according to the double reciprocal plots method of Lineweaver–Burk. In preliminary experiments, the linearity of the reaction with respect to time and enzyme concentration was verified for each PDE preparation.

The test compounds were added to the assay solution containing substrate 10 min before initiating the reaction with the addition of enzyme. By plotting per cent inhibition vs the logarithm of inhibitor concentration, the concentration at which 50 per cent inhibition of the hydrolysis occurred (I_{50}) was determined. The K_i for inhibitors and the nature of inhibition were determined by the methods of Dixon and Lineweaver–Burk.

All the assays were carried out at 30° and performed in triplicate. The test compounds were also examined to ensure that they did not affect the snake venom 5'-nucleotidase or interfere in the radioactivity determination (binding of unreacted [^{3}H]cyclic AMP to the anion exchange resin and liquid scintillation counting). Besides, the underestimation of PDE activity caused by adsorption of adenosine to the resin was evaluated [18]. It was not modified by the test compounds.

Binding studies. Binding studies using unlabeled cyclic AMP and AP 10 to displace protein bound [8-^{3}H]cyclic AMP were carried out with a binding protein prepared from beef skeletal muscle, according to Gilman [19]. This protein kinase binding specifically cyclic AMP was prepared by the procedure of Miyamoto *et al.* [20].

Protein assay. Protein was assayed according to Lowry using bovine serum albumin as standard.

RESULTS

When tested in a wide range of cyclic AMP concentrations (0.125–125 μM), the three PDE preparations exhibited two K_m values as shown by double reciprocal plot representation according to Lineweaver–Burk (Fig. 2). The following low and high K_m were obtained: beef heart, 0.9 and 45 μM; rat heart, 0.6 and 8.3 μM; rat brain, 1 and 50 μM. These results are in general agreement with those found in other studies [11, 12, 21].

The I_{50} values were determined graphically as described in methods, at a cyclic AMP concentration of 0.25 μM for heart preparations and 0.50 μM for brain preparation. The inhibitors concentrations were ranging between 10^{-3} and 10^{-6} M. Then, the

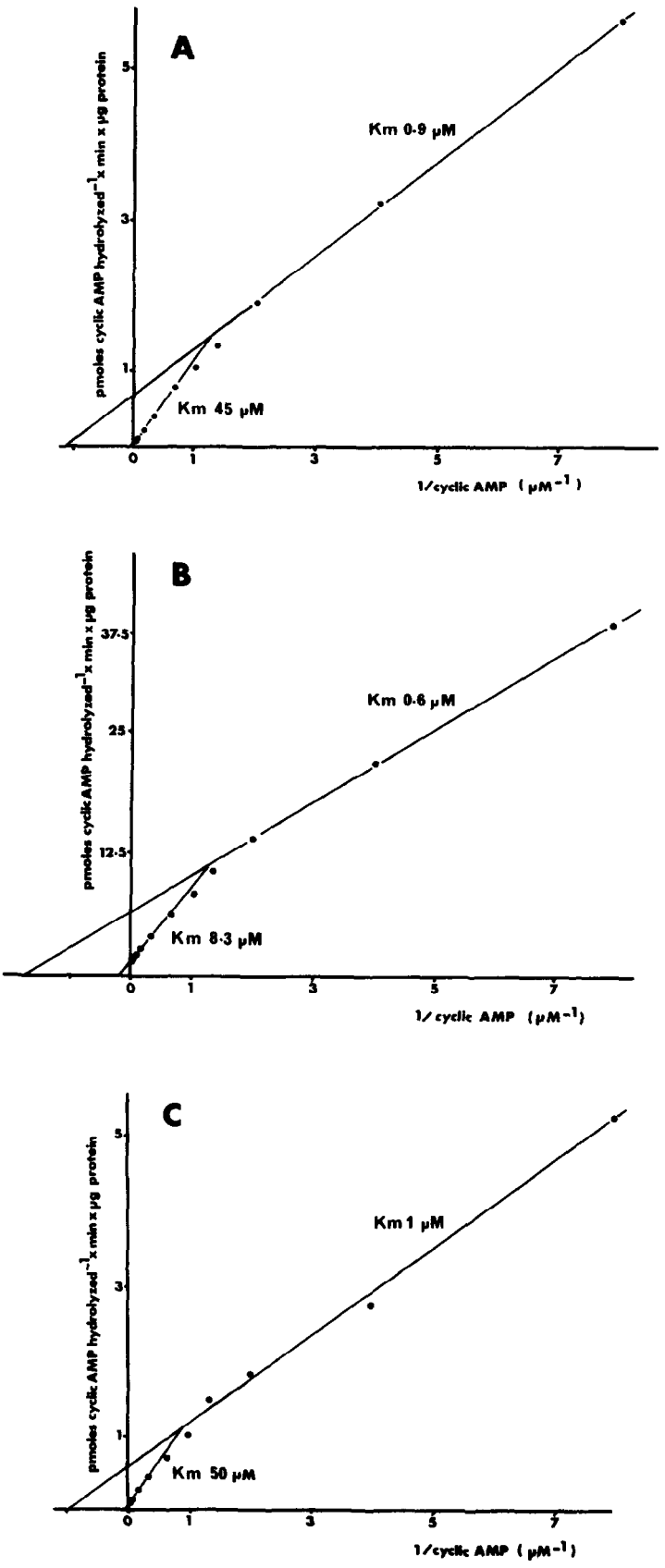


Fig. 2. Cyclic AMP hydrolysis by phosphodiesterase from: beef heart A, rat heart B. rat brain C. Kinetic analysis with double reciprocal plots (Lineweaver–Burk plot) show two K_m.

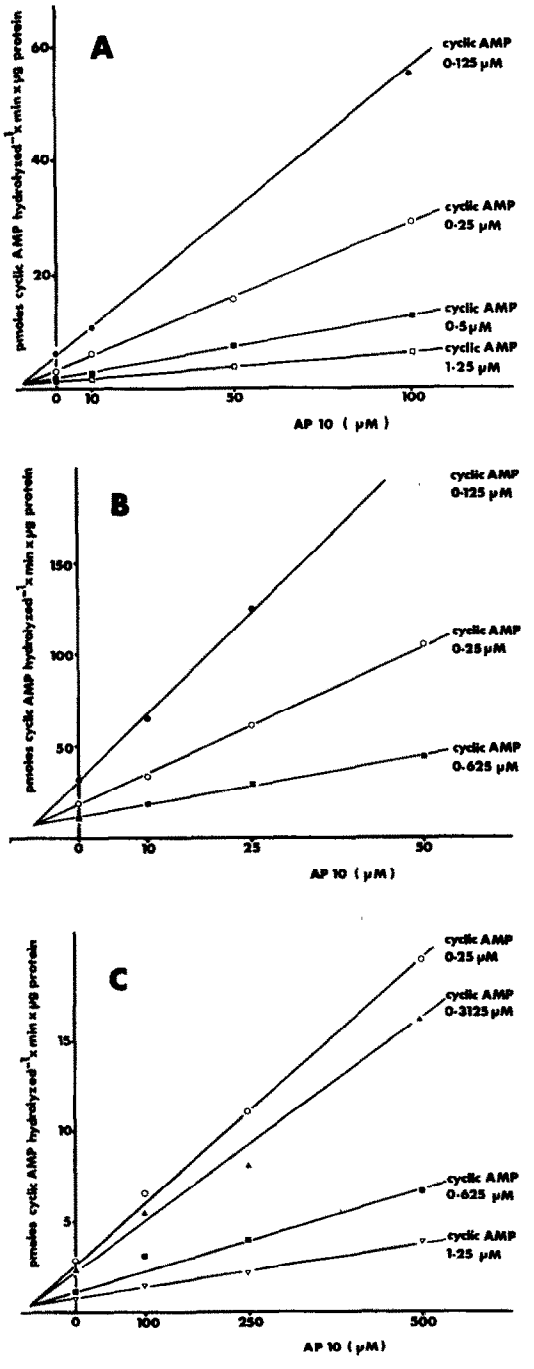


Fig. 3. Dixon plot of AP 10 inhibition of the low K_m cyclic AMP phosphodiesterase from: beef heart A, rat heart B, rat brain C.

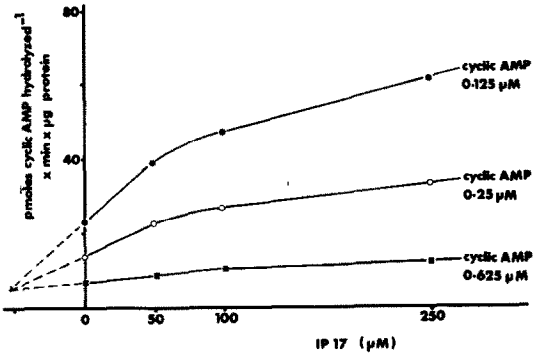


Fig. 4. Dixon plot of IP 17 inhibition of the low K_m cyclic AMP phosphodiesterase from rat heart.

inhibition of the low K_m cyclic AMP phosphodiesterases was investigated by Lineweaver–Burk and Dixon (Figs 3, 4) analysis within a low range of cyclic AMP concentrations (0.125–1.25 μM) and at three concentrations of each test compound. In Table 1, we compare the inhibitory potencies of our cardenolides analogs to that of classical PDE inhibitors: theophylline, MIX, papaverine and to that of the natural standard cardenolide ouabain.

The three cardenolides analogs tested behaved as effective inhibitors of PDEs.

The inhibition produced by AP 10 (Fig. 3) and IP 24 (not shown), like theophylline, MIX and papaverine, appeared purely competitive in both heart and brain preparations. In contrast, the Dixon plots obtained with IP 17 showed a non-classical aspect, concave downward (Fig. 4), with a Hill coefficient [22] less than 1 (see Table 1). In our experimental conditions, the low K_m PDE activity behaved as a Michaelis–Menten enzyme toward substrate. Nevertheless, a negative cooperativity of the purified high affinity PDE for cyclic AMP was shown by Russel *et al.* [23]. Besides, Asano *et al.* [24] obtained Dixon plot similar to that obtained with IP 17 for cyclic GMP inhibition of serum cyclic AMP phosphodiesterase. Beavo *et al.* [25] reported previously that cyclic GMP could act at an allosteric site of cyclic AMP PDE. So, the plot shown in Fig. 4 might reflect a negative cooperativity in IP 17 binding, as observed for effectors of some regulatory enzymes [26].

The most potent inhibitor among the cardenolides tested was AP 10 with K_i of 9, 6.3 and 67 μM for beef heart, rat heart and rat brain respectively. Its relative effectiveness toward cyclic AMP phosphodiesterase was far better than that of theophylline (ten times as effective) and slightly lower than that of MIX and papaverine (three times less effective). In contrast, ouabain, a common standard reference cardenolide, was a poor inhibitor of PDE (25 per cent inhibition at 1 mM). IP 17 was comparable to theophylline. IP 24 was less potent than theophylline.

The K_i of these inhibitors were the smallest for heart PDE and the largest for brain PDE. AP 10 was the most specific among the compounds tested here. Its affinity for the heart enzyme was eight to ten times higher than for the brain enzyme.

In order to investigate the nature of AP 10 inhibition, the beef heart PDE was preincubated in presence of AP 10 up to 2.5 hr at 30°. No rise in inhibition levels was observed. This experiment establishes that the inhibition produced by AP 10 is reversible. This compound, therefore, does not bind covalently to the enzyme in spite of the chemical reactivity of its lactone ring.

The inhibition of beef heart PDE by AP 10 was not reversed by addition of high concentrations of magnesium ions (results not shown) up to 50 mM.

Table 1. Inhibitor constants for cyclic AMP phosphodiesterase from three preparations

		Beef heart PDE	Rat heart PDE	Rat brain PDE
I_{50}, μM*	Theophylline	60	80	178
	MIX	0.9	1.5	5.6
	Papaverine	7.9	1	14
	AP 10	20	40	89
	IP 17	31.5	100	1300
	IP 24	100	250	500
	Ouabain	25 per cent inhibition at 1 mM	Not tested	No effect at 1 mM
K_i, μM†	Theophylline	85	60	190
	MIX	1.4	1.6	2.8
	Papaverine	6	0.54	0.7
	AP 10	9	6.3	67
	IP 17‡	70	55	320
	IP 24	140	140	250

* Measured with a cyclic AMP concentration of 0.25 μM for heart preparatons, of 0.5 μM for brain preparation.

† Measured with cyclic AMP concentrations ranging between 0.125–1.25 μM.

‡ Hill coefficients, calculated according to Lotfield and Eigner [22] with the data of the K_i determination experiments, were: 0.6 for beef heart enzyme (IP 17 concentrations ranging between 5–50 μM), 0.5 for rat heart enzyme (IP 17 concentrations ranging between 50–250 μM), 0.7 for rat brain enzyme (IP 17 concentrations ranging between 50–250 μM). K_i values were assessed by extrapolating the part of Dixon plots corresponding to low inhibitor concentrations.

which indicates that this compound does not behave as a Mg^{2+} chelator.

AP 10 is able to displace cyclic AMP bound to cyclic AMP-dependent protein-kinase from beef muscle, but its affinity for the cyclic AMP binding sites is much lower than cyclic AMP affinity. The relative affinity of the two compounds for the binding protein, as measured in Fig. 5 by the ratio of the slopes [27], is 24,000.

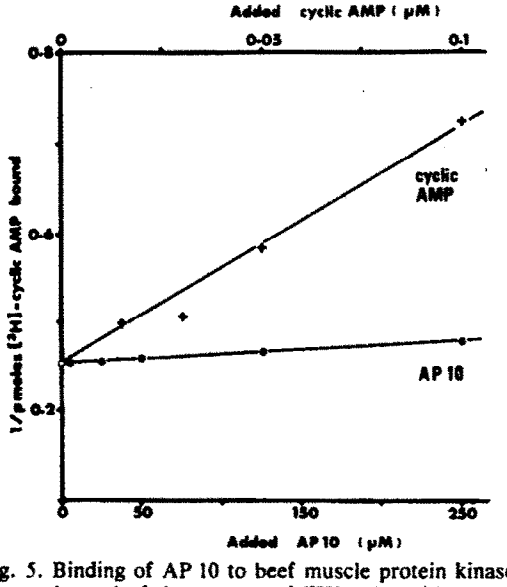


Fig. 5. Binding of AP 10 to beef muscle protein kinase. The reciprocal of the amount of [³H]cyclic AMP bound to protein is plotted against added unlabeled cyclic AMP concentration and added AP 10 concentration. Each assay was performed in 100 μl of reaction mixture which contained 2 μg of binding protein and [³H]cyclic AMP at a saturating concentration of 50 nM.

DISCUSSION

Among the cardenolides analogs considered in this work, AP 10 is the best inhibitor of cyclic AMP phosphodiesterases. Its inhibitory potency is comparable to that of good classical inhibitors such as MIX and papaverine. It is a reversible competitive inhibitor with a good affinity for the heart enzyme ($K_i = 6.3$ μM) and a weaker affinity for the brain enzyme ($K_i = 67$ μM). These results may reflect a preferential action on the cardiac tissue and agree with the observation that AP 10 does not affect the mouse behavior at a dose up to 20 mg/kg (Dr. Legehand, personal communication). Since it is generally agreed that the organ specificity of drugs is related to the difference in sensitivity of the various molecular forms of PDE, our aim will be to investigate the inhibitory potency of AP 10 on separated isoenzymes of heart and brain PDE and to examine its effectiveness on cyclic GMP phosphodiesterase.

The affinity of AP 10 for the cyclic AMP binding sites of bovine protein kinase and the competitive nature of PDE inhibition led us to suppose that AP 10 may behave as an analog of the substrate toward the active site of PDE, in spite of the lack of apparent similitude with cyclic AMP. Synthetic cardenolides with a γ-butyrolactone ring were already shown to inhibit PDEs [8], with a K_i of 60 μM. Another type of compound with an imidazolidinone structure: Ro 7-2956 (and related compounds), which exhibited cardiotonic effects, was also inhibitor of rat erythrocyte PDE [28]. As presented in Fig. 6, the imidazolidinone ring presents some analogy with the butenolide ring of AP 10. Furthermore, it has been shown that ascorbic acid (Fig. 6) whose structure is closely related to cardenolide lactone, is both substrate [29] and inhibitor [30] of phosphodiesterase. So, this struc-

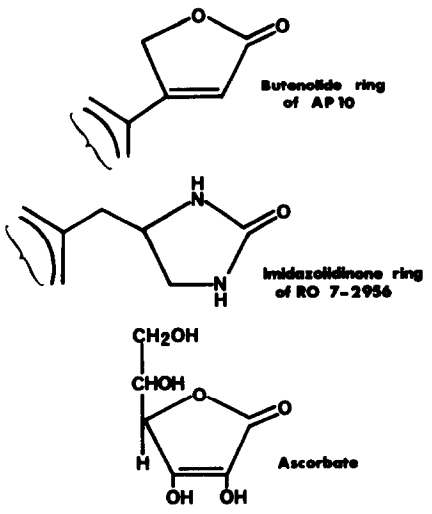


Fig. 6. Structural similarities between the AP 10 butenolide ring, the Ro-7-2956 imidazolidinone ring and ascorbate. These three compounds are inhibitors of cyclic AMP phosphodiesterase.

ture may have an important implication in the occurrence of PDE inhibition.

It is of interest to notice that compounds AP 10 and IP 17 which only differ by the nature of their osidic moiety (Fig. 1) seem to behave differently toward cyclic AMP phosphodiesterase: AP 10 is a classical competitive inhibitor with a Hill coefficient of 1.0 and IP 17 apparently acts with a negative cooperativity.

AP 10 and related compounds may act in cardiac tissue, as Ro 7-2956 and theophylline, by reducing cyclic AMP hydrolysis, and so, raising cyclic AMP level. This could explain their cardiotonic effects, at least partly, and would constitute a quite different way of action with natural cardenolides. Cardiac glycosides, indeed, do not affect PDE but seem to act through Na^+K^+-dependent ATPases inhibition [31–33].

A research of the correlation between effects of AP 10 on the cardiac strength and tissular cyclic AMP level will bring further information on this point.

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