Biochemical Pharmacology, Vol. 31, No. 21, pp. 3353–3358, 1982.
Printed in Great Britain.

0006-2952/82/213353-06 $03.00/0

SELECTIVE INHIBITION OF SEPARATED FORMS OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM RAT HEART BY SOME CARDIO- OR VASO-ACTIVE BUTENOLIDE DERIVATIVES

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(*Received* 15 *December* 1981; *accepted* 9 *April* 1982)

Abstract—Some butenolide derivatives which exhibit pharmacological actions on the cardiovascular system proved to be efficient inhibitors of cardiac cyclic nucleotide phosphodiesterase. Rat heart myocardial enzyme was separated into several distinct forms: three main cytosolic species, isolated by isoelectric focusing and one particulate form, prepared as a washed 105,000 *g* pellet. Butenolidic compounds selectively inhibited a cytosolic form of pI 5.45, which hydrolyses more efficiently cyclic GMP than cyclic AMP at low substrate concentrations, but is preponderant for the hydrolysis of both substrates when they are present at higher concentrations.

Various compounds synthesized in our laboratory and by others, with a butenolide ring fixed on an aromatic moiety, exhibit pharmacological activities on the cardiovascular system: *in vivo* and/or *in vitro* cardiotonic, vasodilating and hypotensive properties [1–4]. We have shown that compounds related to AP 10 (butenolidic deoxybenzoin glucoside) prove efficient inhibitors of cyclic AMP breakdown, more potent *in vitro* on unfractionated heart enzyme than on unfractionated brain enzyme [5], and that this could be involved in their pharmacological action [1]. Myocardial cyclic nucleotide phosphodiesterase has been shown to be of heterogeneous nature by several authors [6]. In previous reports, we described the separation from rat heart of three principal cytosolic forms by isoelectric focusing [7], and of one particulate form [8], differing by their affinities for cyclic AMP and cyclic GMP, by their kinetic properties and their sensitivity to calcium. One cytosolic form, isoelectrically heterogeneous (pI 5.55–6), has a greater affinity for cyclic AMP than for cyclic GMP; it seems to be responsible for the greatest part of cyclic AMP hydrolysis by cardiac cytosol at low substrate concentrations (80–90% at 0.25 μM). It exhibits non-Michaelian kinetics. A cytosolic pI 4.9 form shows a markedly greater affinity for cyclic GMP than for cyclic AMP; it is strongly sensitive to activation by calmodulin plus calcium. It performs nearly 50% of cyclic GMP hydrolysis at low substrate concentrations. Another cytosolic form of pI 5.45 is less specific towards cyclic GMP: it is responsible for nearly 50% of cyclic GMP hydrolysis by cytosol at low substrate concentrations, and is largely preponderant for the hydrolysis of both nucleotides at high substrate concentrations (25 μM). These last two forms show, in our study conditions, Michaelian kinetics [7]. Another phosphodiesterase form identified in a particulate fraction of heart muscle seems to represent a low percentage of total cardiac phosphodiesterase activity. It hydrolyses slightly more cyclic AMP than cyclic GMP when assayed at 0.25 μM of substrate. It exhibits anomalous kinetics with both substrates [8]. In order to state more precisely the interaction between butenolide compounds and cardiac enzymes, we studied the inhibition of separated phosphodiesterase isoenzymes by compounds related to AP 10, and by other pharmacologically active butenolides. Indeed, inhibition studies carried out on crude preparations do not allow an accurate determination of inhibition parameters.

Two reference drugs were also examined: Ro 20-1724, which bears an imidazolidinone ring, presents cardiovascular properties [9, 10], and is described as a cyclic AMP phosphodiesterase specific inhibitor [9, 11, 12]; M&B 22,948, an antiallergic compound related to purines, is described as a cyclic GMP phosphodiesterase specific inhibitor [11, 12].

MATERIALS AND METHODS

Compounds tested. AP10, IP17, IP24 were synthesized in our laboratory by Dr. Prigent [2]. Compound Ro 20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was kindly supplied by Dr. H. Gutmann (Hoffman–La Roche, Basel, Switzerland) and compound M&B 22,948 (2-*O*-propoxyphenyl-8-azapurin-6-one) by Dr. R. Broad (May & Baker Ltd, Dagenham, U.K.). Benfurodil hemisuccinate morpholinium salt (Eucilat®) was generously supplied by Dr. D. Chevaux (Laboratoires Clin-Midy, Lyon, France) and indolinyl butenolide by Dr. J. N. Vallat (Laboratoires Labaz-Sanofi, Toulouse, France).

Materials and enzyme assay. They were previously described in [8]. Briefly, the assay was based on the procedure of Thompson *et al.* [13]; the AG1X2 (Biorad) resin slurry was acidified to improve nucleoside recoveries. These recoveries were systematically evaluated by means of ^{14}C-labelled nucleoside addition to the second step of the assay. Optimal pH and Mg^{2+} concentration were determined for each enzy-

matic form (see Results, for cytosolic forms, and [8] for particulate enzyme) and the assays were performed in these conditions: pI 5.55–6 form, pH 7.5, 5 mM Mg^{2+}; pI 5.45 form, pH 7.7, 7.5 mM Mg^{2+}; pI 4.9 form, pH 7.5, 5 mM Mg^{2+}; particulate form, pH 8, 7.5 mM Mg^{2+}. The extent of inhibition was not significantly different at pH 7.5 or pH 8, in the presence of 5 mM or 7.5 mM Mg^{2+}, as verified on particulate preparation with cyclic AMP as substrate, and on the pI 5.45 cytosolic form with cyclic GMP as substrate. All assays contained 0.5 mg/ml of bovine serum albumin. The proportionality of cyclic nucleotide hydrolysis versus time and protein concentration was checked with all the enzymatic forms. All the compounds were solubilized in Tris–HCl buffer. The absence of interference of the compounds studied with the nucleotidase step of the assay was established.

Separation of phosphodiesterase isoenzymes. The three main cytosolic forms were separated from rat heart 105,000 *g* supernatant fraction by isoelectric focusing on a preparative granular gel plate as described in [7]. The particulate phosphodiesterase preparation was a three times washed 105,000 *g* rat heart pellet, prepared as described in [8].

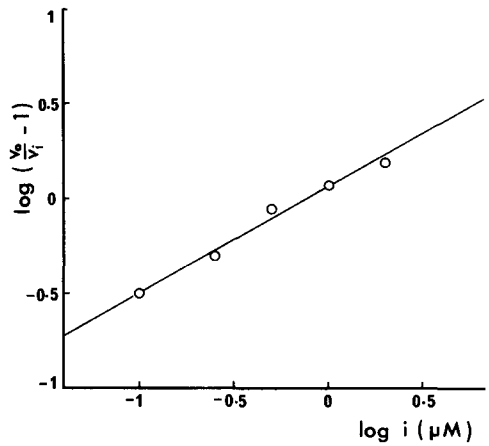


Fig. 2. Inhibition of pI 5.45 phosphodiesterase form by compound M&B 22,948, in the presence of 0.25 μM cyclic GMP as substrate. The data are plotted according to the Hill representation [14]. v_0 = uninhibited rate; v_i = inhibited rate; Hill coefficient (n) is given by the slope of the plot. The value of the ordinate for log $i = 0$ is, in this case (competitive inhibition), $\log \frac{K_m}{K_i (K_m + s)}$, from which K_i value is calculated.

RESULTS AND DISCUSSION

Optimal assay conditions were determined for each cytosolic enzyme form (not shown). The pI 5.55–6 enzyme exhibited a rather broad pH optimum range, the activity being nearly maximal between pH 7.2 and 8.1. Increase in Mg^{2+} concentration was accompanied by an increase in enzyme activity up to 5 mM, for which a plateau was reached. The pI 5.45 phosphodiesterase was maximally active between pH 7.4 and 8. Mg^{2+} addition increased its activity up to 7.5 mM; with higher Mg^{2+} concentrations, activity did not vary. The pH optimum range observed with the pI 4.9 enzyme was very broad: the activity was maximal between pH 6.9 and 7.9. In contrast, the activity of this form presented a sharp peak when it was plotted versus Mg^{2+} concentration: Mg^{2+} increased activity up to 2.5–5 mM, and then higher Mg^{2+} concentrations drastically lowered it; the minimum of activity being reached with 30 mM Mg^{2+}.

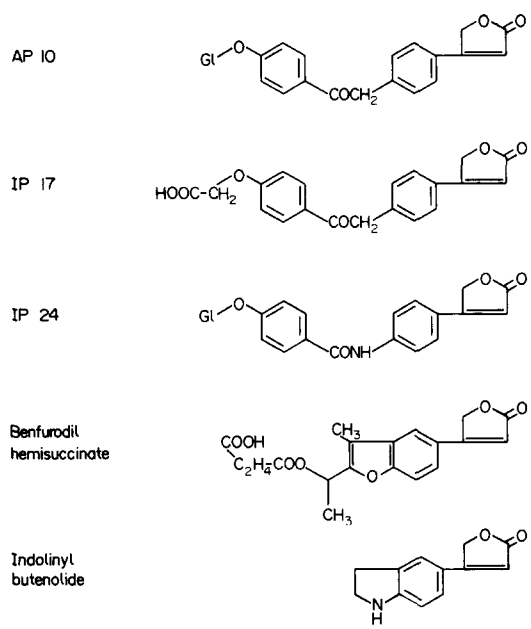


Fig. 1. Structure of butenolide compounds tested as inhibitors of cyclic nucleotide phosphodiesterase.

I_{50} values of the various butenolides (Fig. 1) and of the reference compounds on each phosphodiesterase form were determined at 0.25 μM cyclic nucleotide (in the range of basal physiological concentrations), with the preferential substrate of the considered isoenzyme: i.e. cyclic AMP for pI 5.55–6 form, cyclic GMP for pI 5.45 and pI 4.9 forms, both substrates for particulate enzyme. Inhibition studies were completed with the two most active butenolides (AP 10 and benfurodil hemisuccinate) and the two reference compounds. I_{50} values at high substrate concentration (10 μM, which is probably in the range of the highest cyclic AMP levels physiologically attainable), and kinetic parameters of inhibition were evaluated on the three preponderant enzyme forms (Table 1, Figs. 2 and 3). The pI 5.55–6 cytosolic form assayed in the 0.25–1 μM cyclic AMP range, exhibited linear Lineweaver–Burk plots, corresponding to the 'low K_m' of the enzyme [7]. With the Lineweaver–Burk representation, the inhibition plots of the pI 5.55–6 form were linear and revealed competitive inhibitions, except with compound Ro 20-1724, which proved a non-competitive inhibitor. However, inhibitions by Ro 20-1724, benfurodil hemisuccinate, AP 10, led to concave downward Dixon plots. This might either reflect a heterogeneity of the enzyme, which would be constituted by species with similar specificities toward substrates but with different sensitivities to the drugs, or be related to the non-Michaelian nature

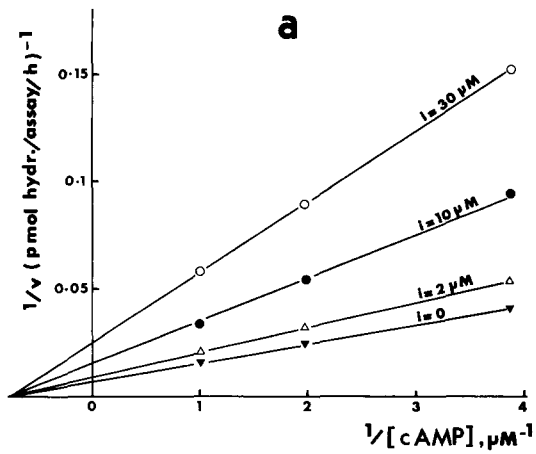
Table 1. Inhibition constants of reference compounds and butenolides on the separated forms of cyclic nucleotide phosphodiesterase from rat heart

Substrate		pI 5.55–6 form cyclic AMP	pI 5.45 form cyclic GMP	pI 4.9 form cyclic GMP	Particulate form cyclic AMP	Particulate form cyclic GMP
I_{50}, μM, at 0.25 μM substrate	M&B 22,948	69	0.7	10.5	126	20
	Ro 20-1724	10	257	519	22	398
	Benfurodil hemisuccinate	24.5	1.8	141	40	57.5
	AP10	51	8	19	36.5	50
	IP 17	400	32	290	1660	575
	IP 24	410	135	190	363	316
	Indolinyl butenolide	115	45	1440	631	398
I_{50}, μM, at 10 μM substrate	M&B 22,948	250	29	24.5		
	Ro 20-1724	33	589	> 2500		
	Benfurodil hemisuccinate	209	56	540		
	AP 10	141	56	91		
K_i, μM*, and Hill coefficient (n)†	M&B 22,948	51	0.8 (0.55)	5.6		
	Ro 20-1724	6 (0.86)‡	126 (0.85)	410		
	Benfurodil hemisuccinate	8.3 (0.68)	1.7 (0.58)	115		
	AP10	9.9 (0.72)	6.6 (0.83)	13		

* K_i were determined with three substrate concentrations (0.25–1 μM for pI 5.55–6 form, 0.25–10 μM for pI 5.45 form, 0.25–4 μM for pI 4.9 form) and four inhibitor concentrations. K_m observed were (in μM ± S.D. in i different experiments): 0.99 ± 0.22 (i = 4) for pI 5.55–6 form, 5.62 ± 1.33 (i = 5) for pI 5.45 form, 1.62 ± 0.38 (i = 4) for pI 4.9 form.

† Lineweaver–Burk plots were linear. When Dixon plots were found to be concave, K_i and n were determined by means of the Hill representation [14]. In the other cases, n was found to be 1 (linear Dixon plots), and K_i were determined by the Dixon method.

‡ In this case, inhibition was of the non-competitive type; in all the other cases, it was of the competitive type.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1/v (p mol hydr./assay/ h)-1
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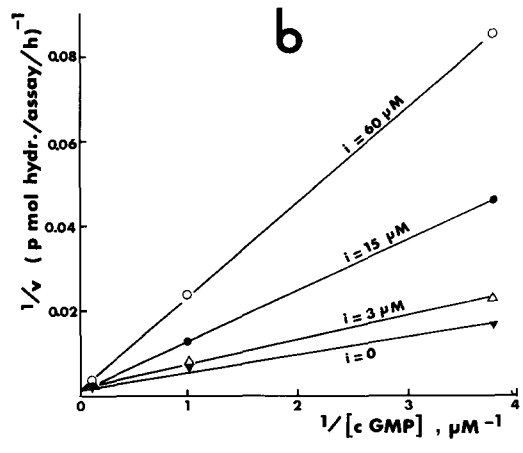
0·1
0·05
i=30 µM
i=10 µM
i=2 µM
i=0
0
1
2
3
4
1/[cAMP], µM-1


b
1/v (p mol hydr./assay/h)-1
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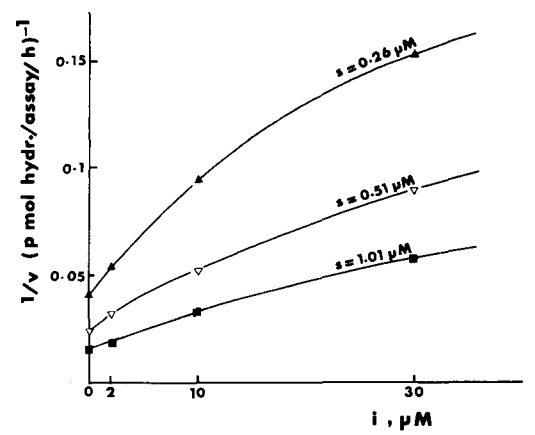
0.06
0.04
0.02
i = 60 µM
i = 15 µM
i = 3 µM
i = 0
0
1
2
3
4
1/[c GMP] , µM -1


1/v (p mol hydr./assay/h)-1
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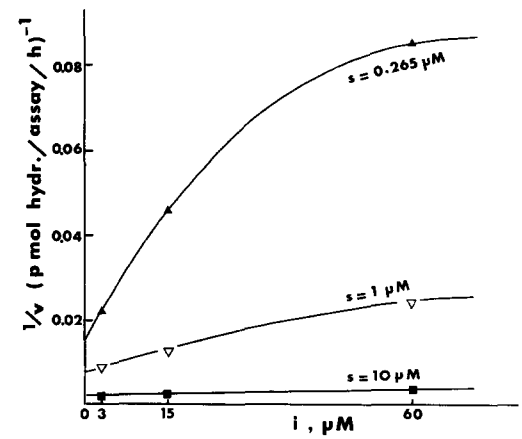
0·1
0·05
s = 0.26 µM
s = 0.51 µM
s = 1.01 µM
0
2
10
30
i , µM


1/v (p mol hydr./ assay / h)-1
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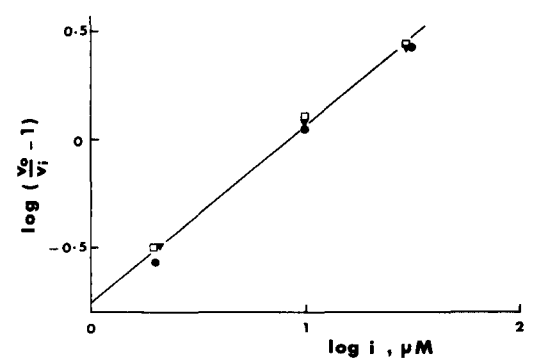
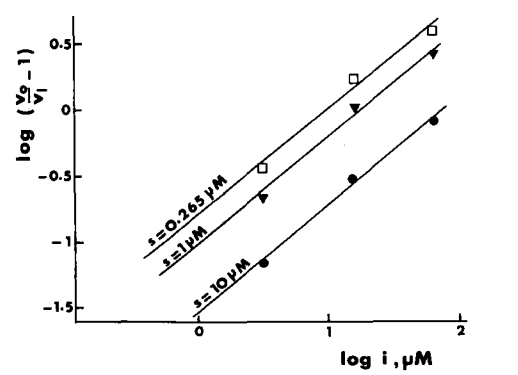
0.06
0.04
0.02
s = 0.265 µM
s = 1 µM
s = 10 µM
0
3
15
60
i , µM


log (Vo/Vi - 1)
0·5
0
-0·5
0
1
2
log i , µM


log (Vo/Vi - 1)
0.5
0
-0.5
-1
-1.5
s = 0.265 µM
s = 1 µM
s = 10 µM
0
1
2
log i , µM

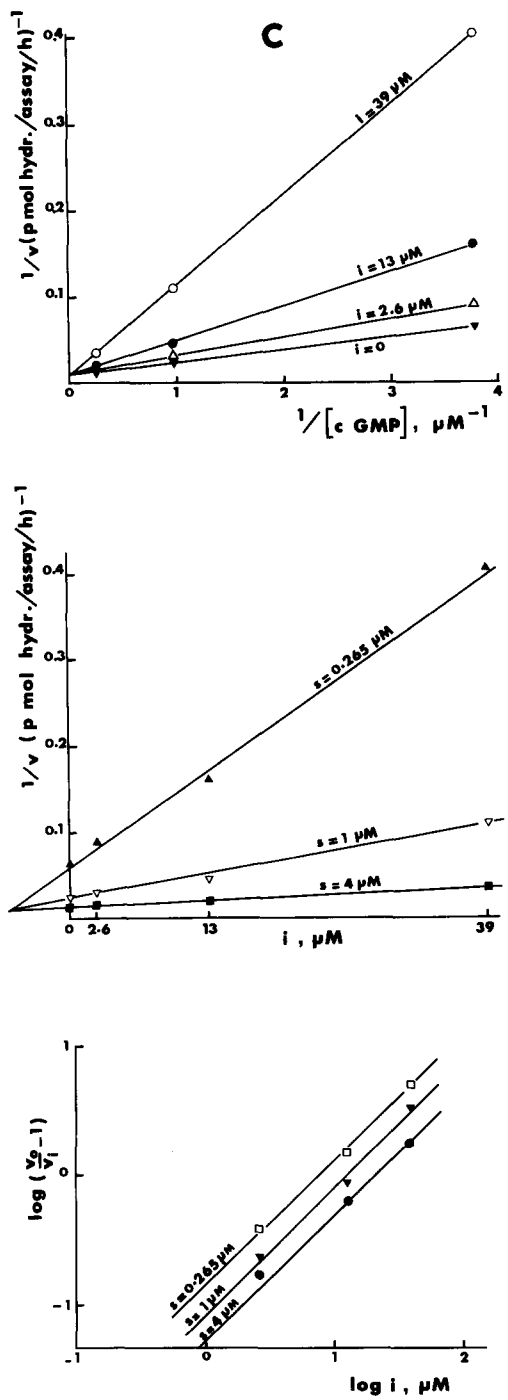


Fig. 3. (a) Inhibition of pI 5.55–6 phosphodiesterase form by compound Ro 20-1724. (b) Inhibition of pI 5.45 phosphodiesterase form by compound AP 10. (c) Inhibition of pI 4.9 phosphodiesterase form by compound M&B 22,948. The same data are plotted according to the Lineweaver–Burk (upper plots), Dixon (middle plots) and Hill methods (lower plots). In the case a, inhibition is of the non-competitive type; with Hill plots, the value of the ordinate for $\log i = 0$ is then $\log \frac{I}{K_i}$ from which K_i value is deduced. In the cases b and c, the inhibition is competitive, K_i is either calculated as in the legend of Fig. 2 (case b), or by means of Dixon plots ($n=I$, case c). In all the cases, n is the slope of the Hill plots.

of the cyclic AMP specific isoenzyme, which exhibits two apparent K_m values when studied in a wide substrate range [7]. K_i and Hill coefficient (n) values were, in these cases, evaluated by means of Hill plots [14]. Coefficient n was found to be inferior to 1, which corresponds to a negative co-operativity. Inhibition studies of cytosolic pI 5.45 and pI 4.9 forms gave rise to linear Lineweaver–Burk plots, all the inhibitions being of the competitive type. With the pI 5.45 form, concave downward Dixon plots were observed, although no evident non-Michaelian property of this form could previously be established. K_i and n were evaluated as described above. We insured that for the pI 5.45 form, the measured K_i of compound AP 10 was not significantly different, whatever the nature of the substrate hydrolysed might be (not shown).

As it is well known that thiol reagents may chemically interact with butenolide ring, the usual presence of 3.75 mM 2-mercaptoethanol in assays might have modified inhibition potencies of butenolide compounds. However, the inhibition exerted by benfurodil hemisuccinate was not lower when measured on a particulate preparation in the presence of a high 2-mercaptoethanol concentration (37.5 mM) than it was in the absence of the thiol.

From these inhibition studies, some interesting points arise. As indicated by I_{50} values measured in the presence of 0.25 μM substrate, specific actions of the drugs under these assay conditions can be pointed out. Compound M&B 22,948, a cyclic GMP phosphodiesterase specific inhibitor in lung [11, 12], is markedly more active on cytosolic forms of pI 5.45 (I_{50} = 0.7 μM) and pI 4.9 (I_{50} = 10.5 μM) which hydrolyse preferentially cyclic GMP at low substrate concentrations, than on the form of pI 5.55–6 (I_{50} = 69 μM). In contrast, Ro 20-1724, a cyclic AMP phosphodiesterase specific inhibitor in erythrocytes, brain, lung [9, 11, 12], is the most potent of the drugs tested in inhibiting the pI 5.55–6 cytosolic form which hydrolyses preferentially cyclic AMP (I_{50} = 10 μM), but has little effect on the pI 5.45 (I_{50} = 257 μM) and pI 4.9 (I_{50} = 519 μM) form. The butenolide compounds are characterized by a striking common feature: they all preferentially inhibit the pI 5.45 cytosolic form with, particularly, a I_{50} of 1.8 μM for benfurodil hemisuccinate, of 8 μM for AP 10. These drugs are respectively 78 and 2.4 fold less potent on the pI 4.9 form, 13.6 and 6.4 fold less potent on the pI 5.55–6 form in these assay conditions. The other butenolides are active on the pI 5.45 form with I_{50} in the 32–135 μM range. Their I_{50} on the pI 5.55–6 form range between 115–410 μM, and between 190–1440 μM on the pI 4.9 form, i.e. notably higher. All the butenolides are less active than Ro 20-1724 on the pI 5.55–6 form, but, in contrast, they are all more active on the pI 5.45 form. The inhibitory potency of butenolides on the membrane-bound phosphodiesterase is rather low (I_{50} equal or superior to I_{50} measured with the pI 5.55–6 form). When I_{50} values are determined at 10 μM substrate, the specificities of the various compounds studied are, at least partly, preserved. Benfurodil hemisuccinate and AP 10 prove, then, nearly 10 times more active than Ro 20-1724 on the pI 5.45 enzyme form (I_{50} of 56, 56 and 589 μM, respectively).

Benfurodil hemisuccinate, commercialized as a peripheral vasodilator and showing some hypotensive and cardiotonic properties [3, 4], proves thus a new potent cyclic nucleotide phosphodiesterase inhibitor, highly specific of one cardiac isoenzymatic form (of pI 5.45), which was not yet reported. AP10 was already described as an efficient phosphodiesterase inhibitor in crude cardiac preparations [5], and was able to increase cyclic AMP level in electrically stimulated left atria isolated from rat heart [1]. The other butenolides have shown cardiotonic and/or vascular effects [2, 3].

In spite of some similarities between the butenolide ring and the imidazolidinone ring of Ro 20-1724, the inhibition patterns observed with the two types of compounds are markedly different. The strongly different affinities of benfurodil hemisuccinate for the pI 5.45 and pI 4.9 enzyme forms (78 fold more potent on the first one at 0.25 μM substrate) underlines the dissimilarity of these two enzymic species, which is not very striking when one considers their respective affinity constants for both cyclic nucleotides [7].

These results agree with the hypothesis that a butenolide ring fixed on an aromatic moiety confers to compounds, beside a cardiovascular action, an inhibitory potency on phosphodiesterase. It is largely accepted that intracellular cyclic AMP level may influence positively rate and force of cardiac contraction [15–17] and vascular smooth muscle relaxation [18, 19]. Compounds that would inhibit *in situ* cyclic AMP phosphodiesterase would contribute to raise this level. Such a mechanism of action has been claimed for several cardiotonic or vasodilatory drugs [9, 10, 15, 18, 20, 21]. However, the butenolides presented in this paper do not inhibit preferentially the cytosolic pI 5.55–6 form, which seems to be responsible, at low cyclic AMP concentrations, for a large part of the hydrolysis of this substrate. On the other hand, they are more potent inhibitors of pI 5.45 form, which is implied in cyclic GMP hydrolysis. So, they probably raise intracellular cyclic GMP levels, which is not incompatible with positive inotropic action and vasodilation, as the implication of cyclic GMP in cardiodepression and vasoconstriction is at present being seriously reconsidered [22–25]. Cyclic GMP would even be the mediator of some smooth muscle relaxant actions [26, 27]. Besides, the pI 5.45 phosphodiesterase plays a major role in cyclic AMP hydrolysis when this substrate is present at higher concentrations, which occurs during hormonal stimulation of cyclase. Phosphodiesterase inhibition by butenolides might thus contribute to cyclic AMP level elevation by potentiation of hormone action, and explain, at least partly, their cardiovascular activities. We hypothesize, particularly, such a mechanism of action for the peripheral vasodilator Eucilat®.

Acknowledgements—This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, INSERM U205 (C.R.L. 81-30-33), and by the Centre National de la Recherche Scientifique, CNRS ERA 560.

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