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MIXED-TYPE INHIBITION OF PULMONARY ANGIOTENSIN I-CONVERTING ENZYME BY CAPTOPRIL, ENALAPRILAT AND RAMIPRILAT

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We have compared at the enzymological level pulmonary angiotensin I-converting enzymes (ACE) purified to electrophoretic homogeneity from four mammalians species: pig, rat, monkey and human. Using both substrates hippuryl-histidyl-leucine and furylacryloyl-phenylalanyl-glycyl-glycine in steady-state conditions, all the ACEs exhibited Michaelis kinetics with identical Michaelis constants, maximal velocities, optimal pH and optimal activating chloride-concentrations. The apparent inhibitory constant was higher for Captopril than for Enalaprilat and even more so for Ramiprilat irrespective of the origin of ACE and the substrate used. Although these inhibitors have been described as competitive inhibitors, Lineweaver–Burk plots were not in accordance with a simple competitive model; moreover, Dixon plots were rather characteristic of non-competitive inhibition. These data emphasize the hypothesis that ACE inhibitors act with mixed-type inhibition, which is consistent with their slow-tight binding to the ACE active center, also with binding of chloride on a critical lysine residue leading to a potential conformational change, and finally with the fact that ACE has two domains, each bearing one catalytic site. On the other hand, as identical kinetic parameters were obtained on the different ACE preparations, results from animal models should allow the extrapolation to humans, in particular for investigations on both renin–angiotensin and kallikrein–kinin systems, and on their inhibition.

Keywords: Angiotensin I-converting enzyme; Enzyme inhibition; Captopril; Enalapril; Ramipril

Abbreviations: ACE, angiotensin I-converting enzyme (peptidyl-dipeptidase, EC 3.4.15.1); HHL, hippuryl-histidyl-leucine; FAPGG, furylacryloyl-phenylalanyl-glycyl-glycine

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INTRODUCTION

Angiotensin I-converting enzyme (ACE) (EC 3.4.15.1) is a key enzyme in both renin–angiotensin and kallikrein–kinin systems by hydrolyzing angiotensin I into angiotensin II, a potent vasoconstrictor and by degrading bradykinin, a vasodilator. This enzyme, mainly localized in the plasma membrane of vascular endothelial cells, has been a major target in the design of antihypertensive drugs. After Captopril, a number of other ACE inhibitors have been synthetized, and most of them are now widely used in the treatment of hypertension, also of cardiac failure, in particular to prevent reinfarction after ischemic heart disease.[1,2] A simple competitive model is usually proposed to account for their inhibition of both plasma and membrane-bound ACEs; but several studies have postulated that ACE inhibitors may act with mixed-type inhibition involving conformational change.[3,4]

In this paper we show that three widely used ACE inhibitors, i.e. Captopril, Enalaprilat and Ramiprilat, which represent three generations of synthetic inhibitors, act as mixed-type competitors for ACE purified from the lung of four mammalian species, i.e. pig, rat, monkey and human, and towards both synthetic ACE substrates hippuryl-histidyl-leucine (HHL) and furylacryloyl-phenylalanyl-glycyl-glycine (FAPGG). We previously verified that all the purified ACEs hydrolyze both substrates under Michaelis conditions and with the same optimal pH and chloride-activations.

MATERIALS AND METHODS

Chemicals

Captopril, Enalaprilat and Ramiprilat were a gift from Bristol Meyers-Squibb (Princeton, NJ), Merck-Sharp and Dohme (West Point, PA) and Hoechst, France (Puteaux), respectively. Enalaprilat and Ramiprilat are the *in vivo* active metabolites (diacids) of Enalapril and Ramipril, both prodrugs being used orally as well as Captopril, but directly active.

Preparation of the Enzyme

ACE was purified to electrophoretic homogeneity by a protocol that we previously detailed.[5] The preparations obtained from porcine (large white pigs), rat (Sprague Dawley), monkey (Cynomolgus Maccacus) and human lungs have been also compared at physicochemical level using chromatographic,

electrophoretic and immunological criterias; in particular, all the purified enzymes showed the same molecular mass of 172 ± 4 kDa on SDS-PAGE.[6]

Enzyme Assays

Kinetic measurements were performed using HHL (hippuric acid as benzoylglycine) and FAPGG in radiochemical and spectrophotometric assays, respectively, as previously detailed.[7,8] HHL is considered as a C-terminal analog of the natural substrate angiotensin I whereas FAPGG is considered as a C-terminal analog of the other main natural ACE substrate, bradykinin. The conditions maintained steady-state kinetics, in particular for the substrate concentrations used, i.e. 1–5 mM for HHL and 0.25–1 mM for FAPGG.

In the radiochemical assay, linearity was maintained during the 60 min of incubation.[9] The liberated ^{14}C-hippurate was extracted and quantified in a liquid scintillation counter as an end-point product.[7]

In the continuous spectrophotometric assay using FAPGG, linearity was also maintained during the 30 min of incubation.[8] The absorbance of ACE did not interfere with the substrate absorbance reading as there was no overlay between both spectra.[10,11]

In both assays, purified ACE was used to start the reaction, thus it was not pre-incubated with the substrates nor the inhibitors; conversely, substrate and inhibitor were mixed and pre-incubated in buffer at 37°C. The final concentrations of ACE protein used were between 0.1 and 0.3 μM for FAPGG and between 1 and 3 μM for HHL. These conditions were chosen in order not to presume of one possible mechanism, involving one or two active sites, identical or not.

RESULTS

In the substrate concentration range covered, in the absence of inhibitor, all the ACEs exhibited Michaelis–Menten kinetics for both substrates; Michaelis constants (K_m), maximal velocities (k_{cat}) and catalytic efficiencies (k_{cat}/K_m) were comparable (Table I). The optimal pH was the same whatever the ACE origin, i.e. pH 8.3 ± 0.05 for HHL and pH 8.2 ± 0.05 for FAPGG. Whatever the substrate, ACE activity always was strictly chloride-dependent with an optimal concentration of 0.375 ± 0.010 M for HHL and 0.30 ± 0.01 M for FAPGG.

The apparent inhibitory constants (K_i) of the synthetic inhibitors were graphically determined from plots of steady-state velocities (v^{-1}) as a

TABLE I Comparison of Michaelis constants (K_m in mM), catalytic constants (k_{cat} in s^{-1}), and catalytic efficiencies (k_{cat}/K_m in $s^{-1} \cdot \mu M^{-1}$) between four pulmonary mammalian ACEs and towards two substrates: HHL and FAPGG. The inter-species discrepancies were not statistically different (each value is within mean ± standard deviation)

	HHL			FAPGG		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
Pig	1.16	650.1	0.56	0.66	2126.4	3.22
Rat	1.16	539.9	0.47	0.66	1632.4	2.47
Monkey	1.16	676.7	0.58	0.66	2211.7	3.35
Human	1.15	601.5	0.52	0.65	1547.6	2.38

function of inhibitor concentration using four concentrations of each substrate. Using HHL, the K_i values were the same towards the four ACEs, but higher for Captopril (20 ± 1 nM) than for Enalaprilat (2.2 ± 0.1 nM) and even more so for Ramiprilat (0.9 ± 0.05 nM); identical data were obtained using FAPGG for Captopril ($K_i = 23 \pm 0.5$ nM), Enalaprilat ($K_i = 2 \pm 0.1$ nM), Ramiprilat ($K_i = 0.75 \pm 0.1$ nM). Between both substrates the difference was statistically significant only for Captopril ($p < 0.03$; Mann–Whitney's U-test). These values of K_i were all in agreement with those calculated by Cheng and Prusoff's method[24] derived from the relationship between K_i and the concentration of inhibitor for 50% inhibition (not shown). Lineweaver–Burk plots obtained with the different inhibitor concentrations did not show an intersection on the $1/v$ axis which is not in accordance with a simple competitive model. Identical patterns were observed for the three inhibitors, tested with both substrates, towards the four purified ACEs and two examples are given in Figure 1 (Captopril with HHL toward pig pulmonary ACE, and Ramiprilat with FAPGG toward human pulmonary ACE). The corresponding Dixon plots (v^{-1} versus inhibitor concentration) were rather characteristic of non-competitive inhibition (Figure 2). Taken all together these data indicate that Captopril, Enalaprilat and Ramiprilat act with a mixed-type inhibition; in particular, they slightly decrease ACE k_{cat} and more largely increase the apparent K_m. Nevertheless, these characteristics allow the direct determination of K_i from Dixon plots.

DISCUSSION

ACE inhibitors were designed to specifically inhibit the generation of the potent hypertensive agent angiotensin II through the action of ACE, the main mechanism of angiotensin II formation of biological relevance.

It is now apparent that at least part of their antihypertensive activity is related to the potentiation of endogeneous bradykinin. Moreover, inhibition of tissue ACE generally has a stronger correlation with the hemodynamic effects of ACE inhibitors than inhibition of ACE in plasma. The efficacy of various ACE inhibitors in hypertensive patients, and also in patients with ischaemic heart disease, might be predicted from differences among the actions of these drugs *in vitro* and in whole animals. Since pharmacologists need extrapolation to the human of data obtained on animal models, it is important to know the degree of homology existing between these species; this we have previously made at both physicochemical and immunological levels.[6] Now we compare the ACEs purified from various mammalians at the enzymological level with the principal focus on synthetic inhibitors. Lung was chosen as the source of ACE because it contains the endothelial isoenzyme only. The pig offers an appropriate model for the study of human vascular diseases potentially treatable by ACE inhibitors, such as thrombosis and atherosclerosis.[12] The rat is used to study the metabolism of glycoproteins, which is not known for ACE, and a number of induced or genetically determined rat models for hypertension have been described.[13] On the other hand, the Cynomolgus Maccacus monkey has been proposed

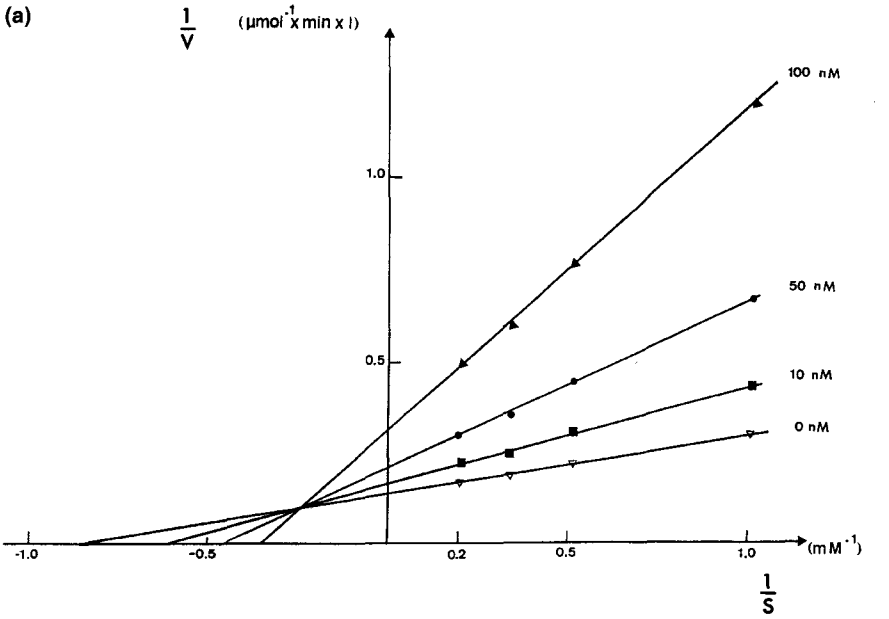

FIGURE 1(a)

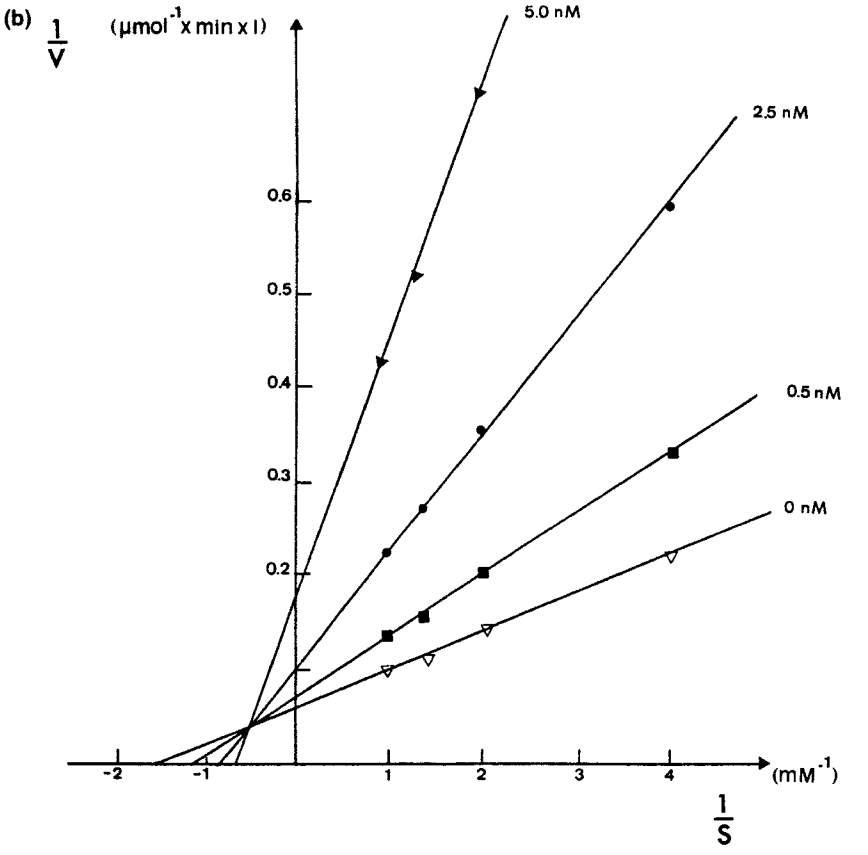

FIGURE 1(b)

FIGURE 1 Lineweaver–Burk plots of inhibition of ACE activity. (a) by Captopril (0–100 nM) with HHL (mM) as substrate towards porcine pulmonary ACE; (b) by Ramiprilat (0–5 nM) with FAPGG (mM) as substrate towards human pulmonary ACE.

as a model for inflammatory pathologies depending on macrophages and resembling the situation in human where macrophages overexpress ACE, such as in sarcoidosis.[14]

First, we verified that, whatever the animal origin of pulmonary ACE, both synthetic substrates HHL and FAPGG were hydrolyzed with identical Michaelis kinetics, maximal velocities, optimal pH and optimal activating chloride-concentrations. These results are in agreement with those of Nishimura *et al.*[15] who worked on human ACE and the same substrates, also with those of Harris and Wilson[16] who compared bovine ACE with the human enzyme, for optimal pH and chloride-activation, as well as for

HHL[17] and FAPGG.[18] This homogeneity at the enzymological level should allow the extrapolation to humans of some data on animal models, namely those we have studied, particularly using FAPGG which is an accurate substrate for kinetic studies on ACE.[19]

Most enzymological studies on ACE inhibitors have been carried out on pulmonary membranes, various ACE extracts or partially purified ACE and have shown that the synthetic compounds Captopril,[20] Enalapril, Lisinopril[21] and Ramipril[22] are competitive inhibitors. From a better understanding of the mechanisms of metalloenzymes with a zinc atom in their active site, such as carboxypeptidases, neutral endopeptidases and peptidyldipeptide hydrolases including ACE,[23] and using better purified ACE preparations, these synthetic inhibitors appeared to exert slow-tight binding to the ACE active center, being transition-state analogs.[24] Moreover, Lisinopril and its dansyl derivative were shown to be mixed-type inhibitors.[3] We now demonstrate that the other ACE inhibitors such as, Captopril, Enalapril and Ramipril also give mixed-type inhibition; these three compounds

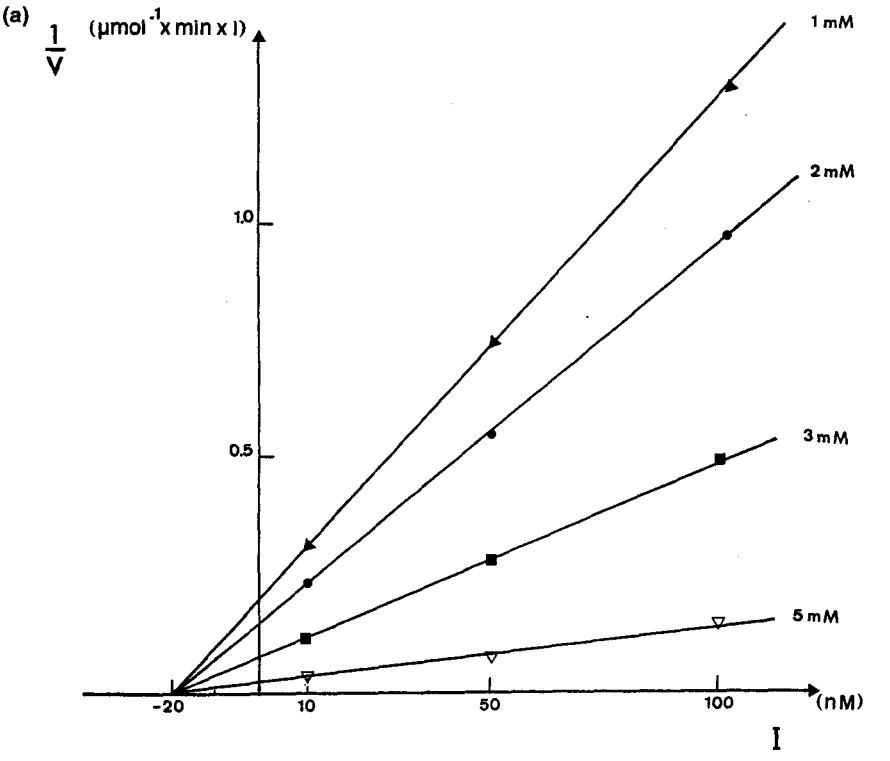

FIGURE 2(a)

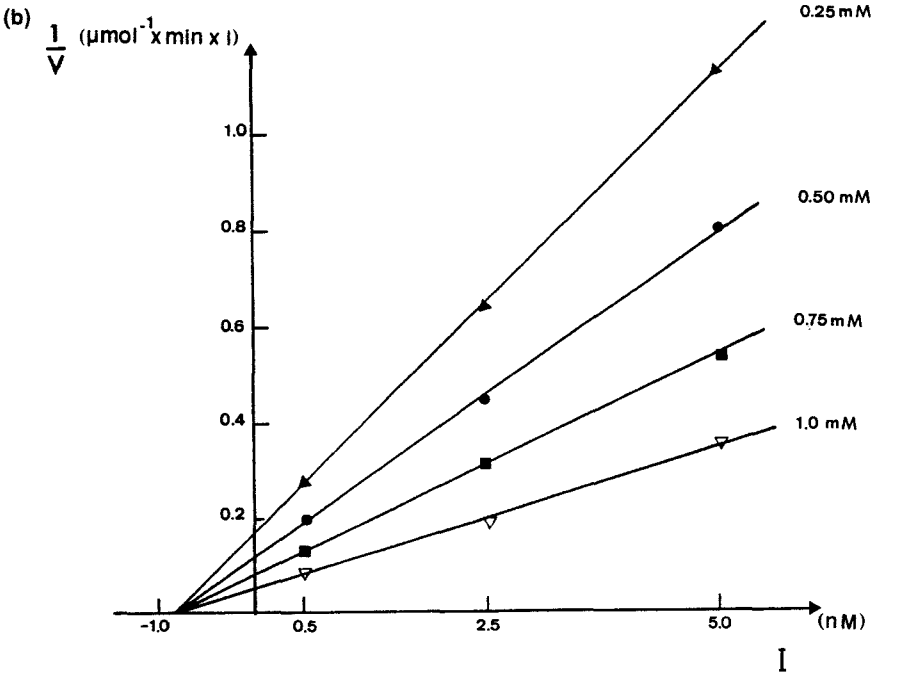

FIGURE 2(b)

FIGURE 2 Dixon plots drawn from the same experiments as in Figure 1. (a) by Captopril (0–100 nM) with HHL (1–5 mM) as substrate towards porcine pulmonary ACE; (b) by Ramiprilat (0–5 nM) with FAPGG (0.25–1 mM) as substrate towards human pulmonary ACE.

represent three generations of ACE inhibitors, i.e. Captopril as the oldest and the unique sulfhydril compound, Enalapril as the first esterified-prodrug and Ramipril as the representative of ACE inhibitors whose relative lipophilicity gives better tissue penetration and binding to ACE. The K_i of $\cong 2$ nM for Enalaprilat obtained with both substrates is higher than that measured by other authors,[21,23] but they used different methods, and these differ in the kind of buffer and ions added, the temperature and pH of incubation, and the dilution of the sample before assay. In particular, Bull *et al.*[21] showed the pH-dependence of the K_i value for Enalaprilat which increases with pH. Nevertheless, at pH 8–9, substrate inhibition is lower than at more acidic pH, in particular with the use of HHL.[25] For Captopril and Ramiprilat, the K_i values are also higher than those previously proposed,[22,26] certainly for the same reasons as above or because of a slow-tight binding inhibition mechanism. On the other hand, all of our values for K_i were in agreement with those calculated by Cheng and Prusoff's method[27] derived

from the relationship between K_i and the concentration of inhibitor for 50% inhibition. In accordance with the literature and here using electrophoretically pure ACEs under strictly identical conditions, Enalaprilat was 10-fold more potent than Captopril, and Ramiprilat was 2 to 3-fold more potent than Enalaprilat. For any one inhibitor, no difference in the K_i value was encountered between species; the K_i value only differed with the nature of the substrate.

Mixed-type inhibition by chemical compounds implies that either a binary complex (enzyme–inhibitor) or ternary complex (enzyme–inhibitor–substrate) undergoes inhibitor-induced isomerization, or conformational modification such as induced by slow-tight binding inhibitors. It is noteworthy that chloride may also bring about a conformational change in a pseudo-allosteric manner[28] involving binding on an ACE lysine residue,[29] an interaction shown by inhibition kinetics.[30] The simplest model is one involving a single inhibitor-binding site; the occupation of this site adversely affects substrate binding so rendering the enzyme incapable of carrying out its catalytic function. A variant of this model involves two mutually exclusive inhibitor-binding sites; binding at the other site gives rise to the non-competitive component of the observed inhibition. This scheme is relevant with reference to the finding that ACE presents two homologous domains,[31] each bearing one fully active site.[32] Thus, it is highly likely that all the known synthetic ACE inhibitors can bind to both sites, but certainly with different inhibition constants and with interference with each other. Unfortunately, both schemes involving conformational change are kinetically indistinguishable under steady-state conditions, as shown for Lisinopril.[3] A better understanding of the alternative modes of inhibition would arise from further studies on the intramolecular dynamics of ACE including mutants with one only active site.

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