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Inhibitory effect of the nonpeptide angiotensin II receptor antagonist losartan and its active metabolite, E-3174, on cAMP phosphodiesterase: additional action of the antagonists

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Abstract—The inhibitory effects of the nonpeptide angiotensin II (AII) receptor antagonist losartan and its active metabolite, E-3174, on bovine brain calcium/calmodulin-dependent 3′:5′-cyclic nucleotide phosphodiesterase (cAMP PDE) were investigated. Losartan and E-3174 inhibited cAMP PDE activity competitively with an apparent K_i of 18.7 ± 2 μM (N = 3) and 70.4 ± 8 μM (N = 3) with respect to cAMP, respectively. With 1.2 mM cAMP as a substrate, cAMP PDE activities were inhibited by losartan and E-3174 in a concentration-dependent manner. The concentrations of losartan and E-3174 required to obtain 50% inhibition of the enzyme activity (IC_{50}) were estimated to be 38.9 ± 7 μM (N = 3) and 139.3 ± 39 μM (N = 3), respectively. These results show that losartan is about four times more potent than E-3174 in inhibiting the enzyme. The Hill coefficient of −1.0 ± − 0.04 (N = 3) for losartan and −1.1 ± − 0.14 (N = 3) for E-3174 was obtained, indicating that one inhibitor binding site is available on cAMP PDE. This study demonstrated that losartan and E-3174 exert additional inhibitory action on cAMP PDE besides AII receptor antagonism.

Key words: angiotensin II; E-3174; losartan; nonpeptide antagonists; cAMP phosphodiesterase

AII* is a biologically active component of the renin-angiotensin system and induces diverse actions which include vascular smooth muscle contraction and aldosterone secretion from the adrenal cortex by binding to specific membrane receptors [1, 2]. The mechanisms of action of AII involve activation of cellular phospholipase C and A_2 via G-proteins [3, 4], stimulation of dihydropyridine-sensitive voltage-dependent calcium channels [5], and inhibition of adenylate cyclase [6]. Activation of phospholipase C increases inositol 1,4,5-triphosphate and diacylglycerol formation, which in turn mobilizes intracellular calcium and activates protein kinase C, respectively [3]. Stimulation of phospholipase A_2 results in a release of arachidonic acid from membrane phospholipids [4] and inhibition of adenylate cyclase results in a decrease in the intracellular levels of cAMP [6].

Losartan, an orally active, selective, nonpeptide and competitive AII receptor antagonist, produces concentration-dependent displacement of AII from AII subtype 1 receptor in vascular smooth muscle [7], inhibits AII-induced contractions in the isolated rabbit aortic strip [8] and lowers blood pressure in furosemide-treated, spontaneously hypertensive or renal hypertensive rats [9, 10]. Losartan, also an extremely long-lasting antihypertensive agent, displays antihypertensive activity quite unlike other antihypertensive compounds such as saralasin and SK&F 108566, suggesting that losartan may have additional actions that are unrelated to AII receptor antagonism [11].

In a study using human astrocytoma, rat C6 glioma or porcine aortic smooth muscle cells in culture [12], it was shown that losartan stimulated the synthesis of prostacyclin, an activator of adenylate cyclase [13]. The inhibition of bovine brain calcium/calmodulin-dependent cGMP PDE by losartan has also been reported [14]. However, further studies are needed to assess the actions of losartan beyond AII receptor antagonism.

In the present study, the inhibition of bovine brain calcium/calmodulin-dependent cAMP PDE by losartan and its active metabolite, E-3174 [15, 16], was investigated.

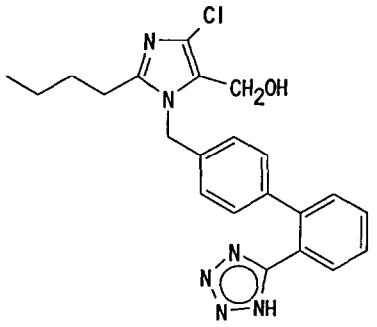
Materials and Methods

Materials. Activator-deficient bovine brain cAMP PDE (affinity purified preparation), bovine brain calmodulin, snake venom 5′-nucleotidase (*Crotalus atrox*), Fiske & Subbarow reducer, cAMP, *p*-nitrophenyl phosphate were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Losartan (2-*n*-butyl-4-chloro-1-[2′-(tetrazol-5-yl)-1,1′-biphenyl-4-ylmethyl]-1*H*-imidazole-5-methanol, potassium salt) and E-3174 (2-*n*-butyl-4-chloro-1-[2′-(tetrazol-5-yl)-1,1′-biphenyl-4-ylmethyl]-1*H*-imidazole-5-carboxylic acid) were products of Merck, Sharp and Dohme Co., Inc. (Rahway, NJ, U.S.A.). All other chemicals were of reagent grade.

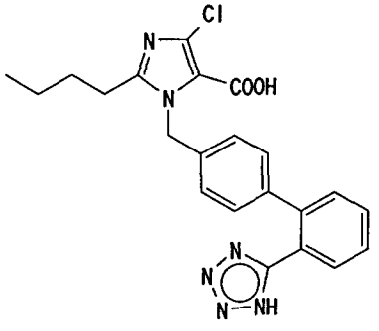
Enzyme assay. Bovine brain calcium/calmodulin-dependent cAMP PDE activity was assayed according to the method of Sharma and Wang [17]. The reaction mixtures consisted of 40 mM Tris–HCl, pH 7.5, 40 mM imidazole, 5 mM magnesium acetate, 0.1 mM calcium chloride, 0.3 U of 5′-nucleotidase, 0.016 U of cAMP PDE, 0.2 μg of calmodulin, and cAMP in a concentration range of 0.02–1.2 mM in a final volume of 0.8 mL. The reaction mixtures were prewarmed for 2 min at 30° and the reaction was started by adding cAMP. The reaction was carried out for 30 min and stopped with the addition of 0.1 mL of 55% trichloroacetic acid. The denatured proteins were removed by centrifugation at 3000 rpm for 10 min and the supernatant (0.5 mL) was withdrawn for the measurement of inorganic phosphorous (Pi). Losartan and E-3174 each at a concentration of 0.1 mM did not interfere with 5′-nucleotidase activity or the chemical measurement of Pi.

Data analysis. Apparent K_m values and dissociation constants of the enzyme–inhibitor complex (K_i) were obtained by fitting substrate-dependent enzyme activities with and without inhibitors to the Michaelis–Menten equation [18] using a nonlinear regression analysis program (GRAFIT, version 2.0, Erithacus Software Limited, U.K.). To correct for substrate utilization, the mean substrate concentrations were used to calculate the kinetic parameters [18]. Using double-reciprocal plots, the nature of inhibition of cAMP PDE by losartan and E-3174 was determined.

* Abbreviations: cAMP, adenosine cyclic 3′:5′-monophosphate; AII, angiotensin II; cGMP, guanosine cyclic 3′:5′-monophosphate; PDE, phosphodiesterase.



Losartan



E-3174

Fig. 1. Chemical structures of losartan and E-3174.

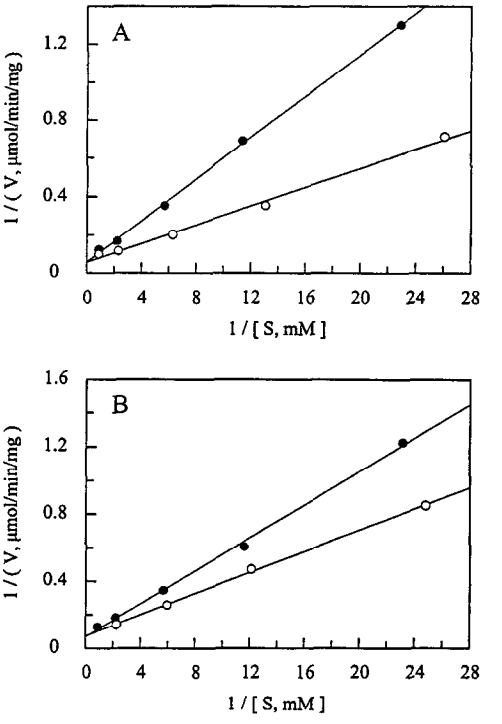


Fig. 2. Double-reciprocal plots showing the competitive inhibition of cAMP PDE activity by losartan and E-3174. Assays were performed as described in Materials and Methods. The enzyme activity (V) is expressed as μmol Pi released/min per mg protein and [S] refers to the concentration of cAMP. Open circles represent the enzyme activity in the absence of inhibitor and closed circles correspond to the enzyme activity in the presence of 0.02 mM losartan (A) or 0.05 mM E-3174 (B). One experiment representative of three performed is shown.

For competitive inhibition, the concentration of inhibitor required to obtain 50% inhibition (IC_{50}) and the Hill coefficients were estimated by fitting the data from concentration-dependent inhibition to the linear form of the Hill equation [18] using the linear least-squares method:

$$\log [E/(100 - E)] = - h \log [I] + \log K'$$

where E is the percentage enzyme activity, I is the concentration of inhibitor, h is the Hill coefficient and K' is a constant equal to IC_{50}.

Results and Discussion

The chemical structures of losartan and E-3174 are shown in Fig. 1. With cAMP as a substrate, bovine brain cAMP PDE reaction followed the Michaelis–Menten kinetics. The K_m value of 0.286 mM (N = 3) was estimated, which is comparable to the K_m values of cAMP PDE from rat brain (0.1–0.3 mM), dog heart (0.49 mM) and bovine heart (0.06–0.1 mM) [19]. When incubated with 0.1 mM losartan or E-3174, 73% or 35% of the enzyme activity was abolished, respectively. The K_i values of 18.7 ± 2 μM (N = 3) for losartan and 70.4 ± 8 μM (N = 3) for E-3174 were estimated, indicating that losartan is about four times more potent than E-3174 in inhibiting the enzyme. Interestingly, whereas losartan displaced AII from the AII subtype 1 receptors competitively [20], E-3174 exhibited noncompetitive AII antagonism [15]. Furthermore, the blocking action of E-3174 is about 41 times more potent than losartan based on pA_2 [15, 20]. Based on ED_{30}, E-3174 is a more potent antagonist than losartan by 20-fold [10, 15]. These findings indicate that losartan and E-3174 interact differently toward cAMP PDE and AII receptors.

Double-reciprocal plots of the inhibition of cAMP PDE by these antagonists indicate that losartan and E-3174 are competitive inhibitors, as can be seen in Fig. 2. For competitive inhibition, the concentration of inhibitor required to obtain 50% inhibition is a function of substrate concentration [18]. With 1.2 mM cAMP as a substrate, the inhibition of cAMP PDE activity by losartan and E-3174 was concentration-dependent with IC_{50} values of 38.9 ± 7 μM (N = 3) and 139.3 ± 39 μM (N = 3), respectively. Figure 3 shows the Hill plots for concentration-dependent inhibition of the enzyme activity by these antagonists. The slopes of the Hill plots were −1.0 ± − 0.04 (N = 3) for losartan and −1.1 ± − 0.14 (N = 3) for E-3174, indicating that cAMP PDE contains one inhibitor binding site.

Recently, Compound I which is a substituted imidazole has been shown to inhibit calmodulin-dependent cGMP PDE from bovine renal artery with a K_i of 5 μM [21]. The inhibition of bovine brain cGMP PDE by losartan with a K_i of 100 μM has also been reported [14]. These data indicate that losartan which also contains the imidazole moiety is 20 times less potent in inhibiting bovine brain cGMP PDE than Compound I in inhibiting bovine renal artery cGMP PDE. However, Compound I was not highly

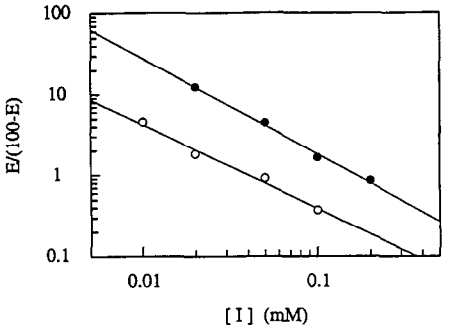


Fig. 3. Hill plots for concentration-dependent inhibition of cAMP PDE activity by losartan and E-3174. Assays were performed as described in Materials and Methods using 1.2 mM cAMP as a substrate. The enzyme activity is expressed as μmol Pi released/min per mg protein and E is the percentage enzyme activity. [I] refers to the concentration of losartan or E-3174. Open circles represent the enzyme activity in the presence of 0.01, 0.02, 0.05 and 0.1 mM losartan. Closed circles correspond to the enzyme activity in the presence of 0.02, 0.05, 0.1 and 0.2 mM E-3174. One experiment representative of three performed is shown.

selective towards bovine renal artery cAMP PDE, as indicated by the IC_{50} value of >200 μM. Thus, losartan is a more potent inhibitor than Compound I in inhibiting cAMP PDE. Losartan is also about five times more selective in inhibiting cAMP PDE than cGMP PDE from bovine brain.

Losartan has been shown to be a very potent AII receptor antagonist with vasodilatory and blood pressure lowering effects [7–10]. A dissociation constant (K_d) of 6.4 nM has been reported for the binding of losartan to AII receptor [22], indicating that losartan inhibits cAMP PDE at a concentration 2900 times more than that required for AII receptor blockade. However, the inhibitory activity of losartan on cAMP PDE is comparable to that of Compound I on cGMP PDE [21]. Also, the IC_{50} value of losartan is similar to those of other selective and potent inhibitors of calcium/calmodulin-dependent cAMP PDE such as phenothiazine (1 μM), vinpocetine (20 μM) and 8-methoxy-methyl-3-isobutyl-1-methylxanthine (4 μM) [23]. The inhibition of cAMP PDE by losartan and E-3174 suggests that these antagonists may contribute in part to antihypertension by increasing the levels of cAMP.

The mechanism of action of cAMP which causes vasodilation is not fully elucidated. However, it has been demonstrated that when cAMP is elevated in cells, cAMP-dependent protein kinase is activated which results in increased protein phosphorylation. The increased protein phosphorylation is thought to be associated with smooth muscle relaxation [24]. Increased protein phosphorylation can also be elicited through inhibition of intracellular protein phosphatases. It was recently reported that AII stimulation of inositol 1,4,5-triphosphate production and calcium signalling in rat hepatocyte was inhibited by okadaic acid [25], a potent inhibitor of protein phosphatase 1, 2A and 2B [26]. The data suggest that this inhibition of AII-induced stimulation is likely to be due to increased protein phosphorylation. On the other hand, when incubated with alkaline and acid phosphatase, or protein phosphatase 2B, losartan and E-3174 inhibited only a small percentage of the phosphatase activities (data not shown), indicating that these antagonists do not have high affinity toward these enzymes.

Activation of phospholipase C by AII causes an increase in inositol 1,4,5-triphosphate and diacylglycerol formation, which in turn mobilizes intracellular calcium and activates protein kinase C, respectively [3]. An increase in the levels of calcium is associated with vascular and smooth muscle contraction [24]. It is, therefore, possible that inhibition of phospholipase C activity may lead to relaxation. Recently, phosphodiesterase inhibitors such as 3-isobutyl-1-methyl-xanthine and rolipram have been shown to inhibit phospholipase C activity [27]. However, phospholipase C activity was not affected by losartan and E-3174 even at concentrations of up to 0.5 mM, suggesting that these antagonists are not involved in the desensitization of calcium signalling pathways.

In summary, losartan and E-3174 were shown to contain additional inhibitory activity on cAMP PDE unrelated to AII receptor antagonism. Further investigation on additional actions beyond AII antagonism of losartan and E-3174 is warranted to gain insight into the mechanisms of long-lasting antihypertensive effect of these antagonists *in vivo*.

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