The Inhibitory Properties of the Bovine Plasma Amine Oxidase Activity by Procaine and Procainamide

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Abstract: The interaction of bovine plasma amine oxidase with local anesthetics, procaine and procainamide has been described. The amine oxidase activity was inhibited by both procaine and procainamide in a mode noncompetitive inhibition when benzylamine and kynuramine were used as substrates. The components of the inhibitors, p-aminobenzoic acid, N,N-diethylaminoethanol and N,N-diethylaminoethylamine which could be produced by the enzyme-catalyzed hydrolysis of the inhibitors in serum failed to exert any effects on the amine oxidase reaction.

Key words: Amine oxidase, Procaine, Procainamide, Kinetic analysis

Introduction

Amine oxidase (amine: O2 oxidoreductase (deaminating, EC 1.4.3.6, AO) is essential to the catabolism and homeostasis of metabolites of biologically important amines. Activity of the oxidase can be followed by monitoring either disappearance of substrates or formation of products. A number of methods based on the direct determination and derivatization of them have been reported[1-6]. A radiochemical method is also available for the sensitive measurement of the AO activity[7-9].

Procaine (Pr) and procainamide (PrA) are familiar anesthetics in clinical and dental therapies. Gerobital H3 with 2% Pr as its ingredient is used to treat the symptoms related to aging and its inhibitory effect on the AO activity has been reported to be time and age dependent[8-10]. The nature of competitive inhibition of the monoamine oxidase (MAO) activity by Pr and its drug Gerobital H3 have been documented using the mitochondrial enzyme from rat-liver[11-13]. While studying bovine plasma AO, we observed that Pr possessed the nature of noncompetitive inhibition toward the enzyme when benzylamine (BzNH2) was used as the substrate.

In this paper, the inhibition properties of Pr and PrA against bovine plasma AO and contribution to the inhibition of their components using both BzNH2 and kynuramine (Kyn) as substrates are described.

Materials and Methods

Materials. Partially purified bovine plasma AO (25 U/mg, EC 1.4.3.6) was purchased from Serva Chemicals (Heidelberg, Germany). Pr·HCl, PrA·HCl, horse radish peroxidase (HRP, 1200 U/mg, EC 1.11.1.7) and horse serum cholinesterase (acylcholine: acylhydrolase, EC 3.1.1.8, >500 U/mg) were from Sigma Chemicals (St. Louis, MO). p-aminobenzoic acid (PABA), N,N-diethylaminoethanol (DEAE-OH)·HCl, N,N-diethylaminoethylamine (DEAE-NH2), BzNH2·HCl, Kyn·2 HBr, homovaleric acid (HVA), 4-hydroxyquinoline (4-HQ) and other chemicals were obtained from Nakarai Chemicals (Kyoto, Japan) and were of reagent grade. Deionized and distilled water was used for preparation of the buffers and reagents.

Hydrolysis of Procaine and Procainamide. Pr·HCl (27.3 mg, 0.1 mmol) and Pr·HCl (27.2 mg, 0.1 mmol) were hydrolyzed in twice-distilled hydrochloric acid at 110°C for 2.5 h. An alternative
method was the serum cholinesterase-catalyzed hydrolysis of Pr (0.1 nmol) and PrA (0.1 nmol) in 0.05 M potassium phosphate, pH 8.0 at 25°C for 24 h according to the method of Kalow. The degree of hydrolysis was followed by monitoring the changes in absorbance and IR spectra. After completion, both hydroxynitrates were lyophilized thoroughly and dissolved in 0.1 M potassium phosphate, pH 7.4. A portion of the solution was used for the AO-inhibition reaction.

Amine oxidase activity.

a. Using BzNH₂ as the substrate. In typical assays for kinetic analysis, to a series of reaction mixtures containing of BzNH₂ (0.05-2 mM, in final concentration), HVA (0.5 mM) and HRP (10 µg/ml), bovine plasma AO (25 µg/ml, in fainal concentration) were added in 0.1 M potassium phosphate, pH 7.4 in the absence and presence of Pr (0.25 mM) and PrA (0.5 mM), respectively. The blank was made in the same way but the BzNH₂ solution was replaced with distilled water. The reaction mixtures were incubated at 25°C for 1 h. Fluorescence intensity was measured against the blank with a Jasco FP-550 spectrofluorometer in a 0.5 cm light-path cell (volume 0.75 ml) using excitation and emission wavelengths of 315 nm and 425 nm, respectively. For time-dependence analysis of the AO activity, the reaction was carried out at 25°C for 0.5-4 hours under the conditions as described in the legend of Fig. 1. The instrument was standardized so that 0.1 µM of quinine sulfate in 0.1 M sulfurous acid gave 1.0 relative fluorescence unit. The calibration graph was made in a similar manner except that the substrate was replaced by various concentrations of hydrogen peroxide (H₂O₂) prepared by serial dilution of the stock. The actual concentration of the stock H₂O₂ was determined by titration with potassium permanganate in 2 M sulfurous acid.

b. Using Kyn as the substrate. The AO activity was measured by quantitating the rate of formation of 4-HQ using Kyn as a substrate. The reaction mixtures in a final volume of 0.25 ml contained various concentrations of Kyn (2-30 pM), bovine plasma AO (25 µg/ml in final concentration) and Pr (20 µM) or PrA (40 µM) in 0.1 M potassium phosphate, pH 7.4. They were incubated at 25°C for 1 hour, after which, 0.5 ml of 0.2 M NaOH was added to the mixture so that the final pH would be about 12. The mixture was heated at 100°C for 3 min and then cooled to room temperature with tap water. Fluorescence intensity of 4-HQ generated was measured against the blank at excitation and emission wavelengths of 312 nm and 382 nm, respectively. The blank was made and incubated in a similar way without Kyn which was mixed after addition of the NaOH solution.

Protein concentration. The concentration of bovine plasma AO was determined from the optical density at 280 nm based on $A_{280}^{1%} = 9.814^{15}$. (data not shown). The presence of Pr and PrA caused serious inhibition of the AO reaction (Fig. 1-a). None or only little effect of PABA and DEAE-OH was observed in the reaction. The presence of DEAE-NH₃ in the reaction mixture caused a little enhancement of H₂O₂ production, possibly due to the presence of the primary amino-group in the compound functioning as a substrate for AO. Pr and PrA are rapidly hydrolyzed by serum cholinesterase in serum. We examined the contribution of the components of Pr and PrA to the inhibition of the AO reaction. Both hydrolysates of Pr obtained by the treatments with acid-hydrolysis and serum cholinesterase had no appreciable effect on the reaction (Fig. 1-b). No remarkable influence of an authentically prepared equimolar mixture of PABA and DEAE-OH was observed in the reaction. The components of PrA, PABA and DEAE-NH₃ did not show any remarkable effects on the AO reaction. The entire molecule of Pr and PrA are thus required to inhibit the AO reaction. The inhibition by Pr and PrA was examined by changes of both the substrate and the inhibitor concentrations. Noncompetitive inhibition was found when BzNH₂ was used as the substrate (Fig. 2). A Dixon plot showed the same noncompetitive inhibition of Pr and PrA on the AO reaction. The Michaelis constant (Km) for the AO reaction using BzNH₂ as a substrate was 0.75 mM and the inhibitor constant (Ki) for Pr and PrA were 0.12 mM and 0.43 mM, respectively.

The linearity of fluorescent intensity against concentrations of 4-HQ was maintained in the range...
Fig. 1 Time-dependence of AO activity in the presence of inhibitors. To a reaction mixture of AO (25 pg/ml, in final concentration), HVA (0.5 mM) and HRP (10 pg/ml) in 0.1M potassium phosphate, pH 7.4, BzNH2 were added at 2 mM concentration in the absence (—–—) and presence of inhibitors. Inhibitors used were a) 5 mM each of Pr (—–£—), PrA (—–¡—), PABA (—–÷—), DEAE-OH (—–Å—) and DEAE-NH2 (—–@—) and b) 5 mM each of the acid-hydrolyzate (—–O—), serum cholinesterase-catalyzed hydrolyzate (—–Å—) of Pr, an authentically prepared equimolar mixture of PABA and DEAE-OH (—–÷—) and 5 mM Pr (—–£—). A mixture without BzNH2 was used as the control. The mixtures were kept at 25°C for 0.5-4 h and fluorescence intensity was measured at excitation and emission wavelengths of 315 nm and 425 nm, respectively.

from 2.5 to 40 μM and 10 μM 4-HQ was found to give 228 relative fluorescence unit under the conditions used. The AO reaction could be followed by the appearance of the fluorescence of 4-HQ using Kyn as the substrate\(^1\). Fluorescent intensity was measured at pH 12 so that the intensity at pH 12 was approximately 10 times higher than that at pH 7.4. The linear dependences of both the time (up to 2 h) and the enzyme concentrations (5-100 μg/ml) were evident in the AO reaction. The AO reaction was dependent on the substrate-concentration; that is, there was linear dependence only in the range 2 to 40 μM. At a substrate concentration exceeding 40 μM, substrate inhibition was observed in the AO reaction under the conditions used. Inhibition of the AO reaction by Pr and PrA was examined by changes in both the substrate and the inhibitor concentrations. Pr and PrA inhibited the reaction in the type of noncompetitive inhibition (Fig. 3), as was also observed by

a Dixton plot. The Km value was 3.8 mM for the AO reaction when used Kyn as a substrate and Ki values were 0.4 mM and 1.2 mM for Pr and PrA, respectively. The present results show the
bovine plasma AO was inhibited by Pr and PrA in the mode of noncompetitive inhibition. For mitochondrial MAO from rat-liver, Pr inhibited reversibly the reaction in the type of the competitive mode\textsuperscript{11-12}. Differentiation in inhibition properties of the two enzymes, inhibitin by other inhibitors such as isoniazid, iproniazid and semicarbazide and substrate-dependence of plasma AO are presently investigated.

**References**

プロカイン及びプロカインアミドによるウシ血漿アミノオキシダーゼ活性の阻害

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局所麻醉剤であるプロカインおよびプロカインアミドのウシ血漿アミノオキシダーゼの活性に及ぼす影響を基質にベンジルアミンおよびキスラミンを用いて調べた。プロカインおよびプロカインアミドの両方とも強くアミノオキシダーゼの活性を阻害し，ともに非競合阻害であった。ベンジルアミンを基質に用いたとき，Km 値は0.75mM で，Ki 値はプロカインに対して0.12mM，プロカインアミドに対して0.43mM であった。

プロカインやプロカインアミドの構成成分である p-アミノ安息香酸，N，N-ジェチルアミノエタノールおよび N，N-ジェチルアミノエチルアミンはアミノオキシダーゼ活性をほとんど阻害しないことがわかった。