Biochemical Pharmacology, Vol. 20, pp. 1967–1971. Pergamon Press, 1971. Printed in Great Britain

# EFFECTS OF CHLOROFORM, DIETHYL ETHER AND A PROPIOPHENONE DERIVATIVE, 3-DIMETHYLAMINO-2-METHYL-2-PHENOXYPROPIOPHENONE HYDROCHLORIDE, UPON CYCLIC 3′,5′-NUCLEOTIDE PHOSPHODIESTERASE*

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(*Received* 3 *August* 1970; *accepted* 11 *September* 1970)

**Abstract**—The effects of chloroform, diethyl ether and 3-dimethylamino-2-methyl-2-phenoxypropiophenone HCl (U-0882) were investigated upon crude cyclic 3′,5′-nucleotide phosphodiesterase prepared from dog heart. Chloroform noncompetitively inhibited the enzyme at a partial pressure close to the clinical application. The partial pressure which gave a half-maximal inhibition was estimated to be 30 mb. Diethyl ether, on the other hand, inhibited the enzyme preparation when its partial pressure was above the clinical application and a half-maximal inhibition was induced at 425 mb. The enzyme preparation was inhibited by U-0882 competitively. The concentration which inhibited the enzyme half maximally was about 0·9 mM. A possible role of cyclic AMP in mediating the arrhythmogenic effect of catecholamines is discussed.

IN RECENT years evidence has accumulated indicating that the beta-effect of catecholamines upon myocardium may be mediated through the intracellular increase of cyclic adenosine 3′,5′-monophosphate (cyclic AMP). This possibility was reviewed extensively by Sutherland *et al.*[1] Catecholamines stimulate adenyl cyclase, which synthesizes cyclic AMP from ATP. Cyclic AMP is in turn hydrolyzed to adenylic acid by cyclic 3′,5′-nucleotide phosphodiesterase (phosphodiesterase). Theophylline and caffeine inhibit the latter enzyme. In the presence of these inhibitors, increased hormone actions were reported.[1]

Chloroform is known to increase the incidences of arrhythmias associated with the administration of epinephrine. This myocardial sensitizing action was also reported with the administration of a propiophenone derivative, 3-dimethylamino-2-methyl-2-phenoxypropiophenone HCl (U-0882).[2] The present study was undertaken to see if these agents might inhibit phosphodiesterase prepared from dog hearts.

## EXPERIMENTAL PROCEDURE

Crude cyclic 3′,5′-nucleotide phosphodiesterase was prepared from dog hearts according to the method of Nair[3] with the following modifications. Dogs were anesthetized with intravenous pentobarbital and hearts were removed after exanguination from the femoral artery. After removal of valves and residual blood, hearts were kept at −20° until use. The following steps were performed in a cold room. The frozen heart was sliced and homogenized with 5 vol. of distilled water, which contained

* This study was supported by a Clinical Investigatorship from the Veterans Administration Hospital.

0·05% (v/v) Triton X-100, in a Waring blender for 3 min at the highest peed. The homogenate was stirred for 30 min, then centrifuged at 5000 *g* for 15 min. The supernatant solution was filtered through four layers of surgical gauze with a Buchner funnel. The first ammonium sulfate fractionation, ethanol fractionation and second ammonium sulfate fractionation were performed according to the original method. The second ammonium sulfate fraction was chromatogramed upon a Sephadex G-200 column which was equilibrated with 10 mM tris–HCl, pH 8·0, and eluted with 50 mM tris–HCl, pH 8·0. The active fraction appeared in a single peak right after the column dead space. The eluate was used in this study without further purification.

The activities of phosphodiesterase were determined by the method of Butcher and Sutherland.[4] The assay mixture consisted of an appropriate amount of cyclic AMP, 5 mM $MgCl_2$, 50 mM tris–HCl, pH 8·0, and 0·5 mg snake venom (*Crotalus atrox*) in a total volume of 2·1 ml. In a Warburg flask, the enzyme preparation was placed in the side arm and the substrate mixture was placed in the main flask and equilibrated for 10 min with the anesthetics. Then they were mixed and incubated for 30 min at 38° in a Dubnoff shaker. Anesthetics were maintained in a gas phase during the incubation period. Chloroform and diethyl ether were vaporized with a Copper Kettle and diluted with oxygen. The concentrations of anesthetics were estimated by the kettle temperature and the flow of the diluent gas. The accuracy of the concentration was checked with a Hitachi Perkin–Elmer K23 gas–liquid chromatograph. Anesthetic concentrations were expressed as its partial pressure in millibars (1000 dyn/cm$^2$) in the gas phase equilibrated with the reaction mixture. U-0882* was dissolved in the substrate mixture.

The reaction was terminated by the addition of 0·1 ml of 80% (w/v) trichloroacetic acid, chilled, and the liberated inorganic phosphate was determined by the molybdovanadate method reported elsewhere.[5] The presence of U-0882 formed turbidity by the addition of molybdate reagent. Therefore, U-0882 was removed from the reaction mixture with chloroform before the inorganic phosphate assay. The protein concentration was determined by the method of Lowry *et al.*[6]

## RESULTS

Inhibition of the enzyme activity with chloroform was seen when its partial pressure exceeded 5–10 mb. The partial pressure of diethyl ether, which showed apparent suppression of the phosphodiesterase activity, was above 100 mb. When the reciprocals of the cyclic AMP concentrations were plotted against the reciprocals of the reaction velocity, noncompetitive inhibitions were demonstrated with chloroform and diethyl ether (Figs. 1 and 2). The apparent inhibitor constants were estimated graphically by constructing a Dixon plot and were found to be 30 mb for chloroform and 425 mb for diethyl ether. Figure 3 shows a Lineweaver–Burk plot of U-0882. A competitive inhibition was observed. The apparent inhibitor constant was estimated to be about 0·9 mM.

## DISCUSSION

Robison *et al.*[7] and Williamson[8] demonstrated that the inotropic action of epinephrine is mediated through the increase of cyclic AMP in the myocardium. After

* We are indebted to Dr. W. A. Freyburger, Upjohn Company, who supplied the U-0882 used in this study.

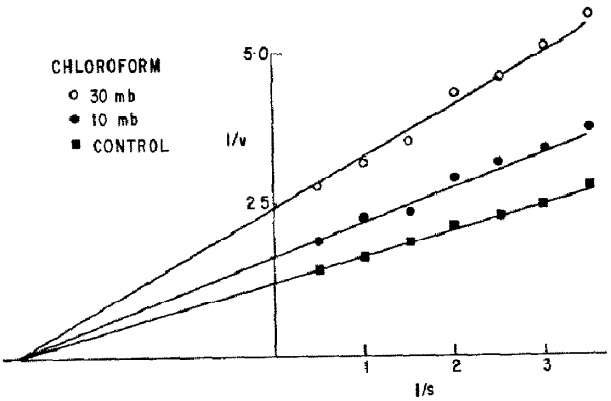


FIG. 1. Lineweaver–Burk plot of chloroform inhibition of phosphodiesterase. Ordinate: reciprocal of reaction velocity expressed as $10^{-6}$ moles inorganic phosphate released per mg of protein per min. Abscissa: reciprocal of cyclic AMP concentration (mM). The concentrations of chloroform are expressed by the partial pressures in the gas phase equilibrated with the reaction mixture, and were 10 and 30 mb. A noncompetitive inhibition was found.

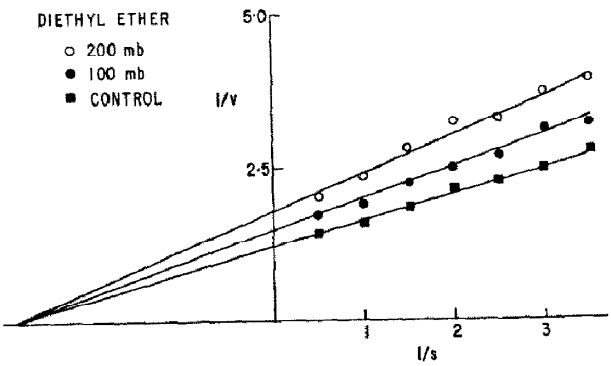


FIG. 2. Lineweaver–Burk plot of diethyl ether inhibition of phosphodiesterase. See legend of Fig. 1. The partial pressures of diethyl ether in the gas phase were 100 and 200 mb. A noncompetitive inhibition was found.

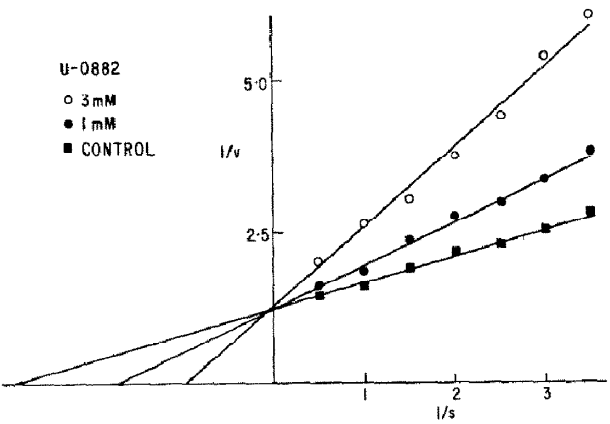


FIG. 3. Lineweaver–Burk plot of U-0882 inhibition of phosphodiesterase. See legend of Fig. 1. The concentrations of U-0882 were 1 and 3 mM. A competitive inhibition was found.

epinephrine administration, the level of cyclic AMP in the myocardium reached maximum within 4 sec and the maximum increase of myocardial contractile force appeared more than 20 sec later. The possibility that cyclic AMP may transmit beta-receptor agonistic action of catecholamines, such as inotropism and chronotropism, has been reviewed.[1] Although bathmotropic effects of sympathomimetic amines during anesthesia with the so-called myocardial-sensitizing agents appear to involve a beta-receptor, their relationships with cyclic AMP have not been reported to our knowledge.

Papp and Szekeres[9] reported that the arrhythmogenic action of catecholamines occurred in two phases. The first phase was characterized by the decreased threshold of electrical fibrillation and lasted only 8–30 sec. The arrhythmia was due to a stimulation of beta-receptor and was counteracted by beta-receptor blocking agents. Administration of alpha-receptor blocking agents were without effect. In the second phase, the arrhythmia was induced through elevation of the blood pressure by alpha-action of catecholamines. The threshold of electrical fibrillation was elevated in this phase. This arrhythmia can be antagonized by inhibiting the rise in blood pressure. The administration of beta-receptor antagonists was not only ineffective, but sometimes increased the incidence of the arrhythmias. The occurrence of arrhythmia in the first phase was enhanced by the administration of chloroform and cyclopropane, and frequently led to fatal ventricular fibrillations. The arrhythmias of the second phase consisted of scattered ventricular extrasystoles, which did not lead to ventricular fibrillation. This short-lived first phase arrhythmogenic action of epinephrine may have some relevance to the short-lived elevation of cyclic AMP level in the myocardium described by Robison *et al.*[7] and Williamson.[8]

Since theophylline and caffeine inhibit the hydrolysis of cyclic AMP and catecholamines transmit their signal by the increased biosynthesis of cyclic AMP, the enhanced action of catecholamines is expected in the presence of these inhibitors. Enhancement of hormone action by these agents is taken as one of the criteria which implicate cyclic AMP as an intermediate messenger.[1] Rall and West[10] reported that the inotropic response of catecholamines is enhanced by theophylline on isolated atrial strips. McNeil *et al.*[11] concluded that the inotropic action of theophylline *per se* may not be related to its inhibitory action upon phosphodiesterase, but the potentiation of catecholamines might have relevance to the phosphodiesterase inhibition. Ueda *et al.*[12] reported that the administration of theophylline or caffeine enhanced the bathmotropic action of epinephrine in dogs, leading to fatal ventricular fibrillations in most cases.

The present results suggest that the increased susceptibility to epinephrine fibrillation by myocardial sensitizing agents may be related in some way to the decreased rate of the removal of cyclic AMP. The apparent inhibitor constant of diethyl ether was in excess of clinically applicable concentrations, whereas that of chloroform showed a value which was not too far from the clinical application. The reported dose of U-0882 which increased the arrhythmogenic effect of epinephrine was 10 mg/kg in dogs.[2] The apparent inhibitor constant of U-0882 was about 30-fold in excess of this dose. Although the extractability of U-0882 into organic solvent suggests that this agent may be concentrated into cell lipids, the distribution of this drug in the body is unknown. The comparison of its concentrations *in vitro* and intravenous doses per kilogram of body weight may not be relevant.

Aside from methylxanthines, a variety of compounds have been reported to inhibit phosphodiesterase: sulfonylurea derivatives,[13] benzothiadiazine derivatives,[14,15] puromycin,[16,17] papaverine,[17] ATP and inorganic polyphosphate,[18] and phenothiazine derivatives and other tranquilizers.[19] Hollister and Kosek[20] reported sudden deaths occurring among patients receiving heavy doses of tranquilizers. In one patient, who was on chlorpromazine and thioridazine medication, the death was attributed to recurrent ventricular fibrillation. Whether this has relevance to our finding remains to be established. Studies of cardiac effects and epinephrine interaction of these agents listed above may be helpful in evaluating our results.

Another criterion which would implicate cyclic AMP as a second messenger of hormone action is an appropriate response of the level of cyclic AMP to hormonal stimulation in the target organ.[1] However, because of a possible compartmentalization of this compound in the cell, and because ventricular automaticity appears to originate from the specialized conductive tissue,[21,22] the gross level of cyclic AMP in the heart may not reflect the bathmotropic activity of epinephrine. Measurement of the cyclic AMP content of subcellular fractions of the specialized tissue may be indicated. Myocardial automaticity induced by epinephrine is a complicated bioelectrical phenomenon influenced by many factors and its relation to metabolism requires future study.

*Acknowledgement*—The authors wish to thank Dr. N. A. Bergman for his help in preparing this manuscript.

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