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ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE PHOSPHODIESTERASE IN GUINEA-PIG LUNG—PROPERTIES AND EFFECT OF ADRENERGIC DRUGS*†

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Abstract—The hydrolysis of adenosine 3′,5′-cyclic monophosphate (cAMP) by adenosine 3′,5′-monophosphate phosphodiesterase (PDE) was studied in whole homogenates and cell fractions of guinea-pig lung. Approximately 60 per cent of the activity of the whole homogenate was contained in the 10,000 *g* supernatant. This cell fraction contained PDE with two separate affinities for cAMP: PDE I with low affinity $K_m \simeq 3{\cdot}1 \times 10^{-4}$ M; $V_{max} \simeq 90$ nmoles cAMP hydrolyzed/mg protein/20 min and PDE II with high affinity $K_m \simeq 5{\cdot}7 \times 10^{-5}$ M; $V_{max} \simeq 35$ nmoles cAMP hydrolyzed/mg protein/20 min. Both forms required magnesium which could be replaced by manganese and cobalt but not by copper. Aminophylline and theophylline inhibited both PDE forms. Calcium only inhibited PDE I but not PDE II. Adrenergic drugs inhibited PDE I and at 0·1 mM decreased in potency in the following order: isoproterenol = epinephrine > norepinephrine > methoxamine. Butoxamine was ineffective on PDE I. Only norepinephrine inhibited PDE II but not to the same extent as its effect on PDE I. Chelation of added magnesium and endogenous calcium was not responsible for the inhibition of PDE I by the adrenergic drugs used. Propranolol (0·1 mM) and phentolamine (0·05 to 0·001 mM) alone did not effect PDE I. Propranolol (0·1 mM) failed to reverse the inhibitory effect of isoproterenol (0·1 mM) and epinephrine (0·1 mM). Thus a β-adrenergic mechanism could not explain the inhibitory effect of these agents. Phentolamine (0·05 to 0·001 mM) reversed the inhibition by epinephrine bitartrate but not by epinephrine hydrochloride. These data are inconclusive to support an α-adrenergic mechanism for the inhibition of cAMP phosphodiesterase by epinephrine.

ADENOSINE 3′,5′-cyclic monophosphate (cAMP) has been implicated as a controlling agent in a number of processes which govern lung function. Thus increases in cAMP have been associated with tracheal smooth muscle relaxation[1] and decreases with the immunological release of histamine from human lung.[2] The physiological responses of these systems and the intracellular cAMP levels respond to adrenergic stimulation via both α- and β-receptors.[2,3]

Intracellular levels of cAMP may be modulated by alteration of the basal activities of either adenyl cyclase or adenosine 3′,5′-cyclic monophosphate phosphodiesterase (cAMP phosphodiesterase). Adenyl cyclase has frequently been associated with the β-adrenergic receptor.[4] Recently Amer[5] reported that epinephrine increased the

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affinity of rat liver cyclic nucleotide phosphodiesterase for cAMP and speculated that this might be via an α-adrenergic mechanism.

This report describes the results of experiments which demonstrated that guinea-pig lung contains two forms of phosphodiesterase with different affinities for cAMP and that predominately the low affinity form is affected by adrenergic drugs.

MATERIALS AND METHODS

Chemicals. Unlabeled 3′,5′-cAMP, aminophylline, L-epinephrine hydrochloride, L-epinephrine bitartrate, L-isoproterenol hydrochloride, L-propranolol hydrochloride and *Crotalus atrox* snake venom were obtained from the Sigma Chemical Company, St. Louis, Mo. Methoxamine hydrochloride and butoxamine hydrochloride were gifts from the Burroughs Wellcome Company. Phenoxybenzamine was a gift from Smith, Kline & French. Norepineprine bitartrate was obtained from Winthrop Laboratories, N.Y.; phentolamine mesylate, from the Eli Lilly Company and theophylline from Nutritional Biochemicals. Tritiated 3′,5′-cAMP (sp. act. 28 Ci/m-mole) was obtained from the Schwartz Mann Radiochemical Company, Orangeburg, N.Y.

Preparation of enzyme. Adult female Hartley guinea-pigs (250–350 g) were killed by a blow on the head, exsanguinated and the lungs immediately removed. The lung tissue was separated from the major airways and blood vessels and homogenized with a Potter–Elvehjem homogenizer, at 4° in nine parts of Tris–HCl buffer, pH 7·5, containing 0·25 M sucrose. The homogenate was filtered through a cheese cloth and the filtrate was centrifuged in a refrigerated Sorvall model RC-2-B centrifuge at 1000 *g* for 6 min. The 1000 *g* supernatant was centrifuged at 10,000 *g* for 40 min. The particulate fractions and aliquots of the 10,000 *g* supernatant were kept frozen at −20° until required for use. Under these conditions the enzyme remained stable for at least 3 months. All the enzyme preparations were derived from the pooled lung homogenates from three to six animals. Protein determinations on the various fractions were carried out according to the method of Lowry *et al.*[6]

Incubation procedure. Cyclic AMP phosphodiesterase activity was determined essentially by the method of Beavo *et al.*[7] For routine assays the incubation mixture contained 50 μmoles of Tris–HCl buffer, pH 7·5, 5 μmoles of magnesium chloride, 500,000 dis/min ^{3}H-3′,5′-cAMP, and substrate, drugs and ions as indicated, in a volume of 1 ml. The reactions were carried out at 30° for 20 min and started by the addition of 0·1 ml of 10,000 *g* supernatant. The reaction was terminated by placing the tubes in a boiling water bath for 75 sec. Tissue and substrate blanks were incubated at the same time. The ^{3}H-3′,5′-AMP formed was converted to ^{3}H-adenosine by the addition of 0·2 ml of 0·2% (w/v) *C. atrox* venom 5′-nucleotidase and incubating at 37° for 10 min. Separate experiments established that this was sufficient time for complete conversion to take place. Control experiments were carried out to establish that the results to be described were not due to an effect on the 5′-nucleotidase.

Assay of ^{3}H-adenosine. The ^{3}H-adenosine was separated from the other tritiated components in the mixture by the method of Huang and Kemp[8] and determined by liquid scintillation spectrophotometry. All samples were corrected for quenching by the use of an internal standard. The data to be described are expressed as nanomoles of 3′,5′-cAMP hydrolyzed per milligram of protein per 20 min and are the result of duplicate incubations from at least three separate experiments.

RESULTS

Properties of guinea-pig lung phosphodiesterase. Intracellular distribution, tissue and time linearity studies were conducted with a substrate concentration of 1 mM. Table 1 shows the intracellular distribution of enzyme activity. About 60 per cent of the activity of the whole homogenate was observed in the 10,000 *g* supernatant. Figure 1

TABLE 1. INTRACELLULAR DISTRIBUTION OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE IN GUINEA-PIG LUNG

	3′,5′-cAMP (nmoles hydrolyzed/10 mg tissue*/20 min; $n = 3$)	Whole homogenate (%)
Whole homogenate	109·3 ± 6·92†	
1000 *g* Precipitate	29·94 ± 1·74	27
1000 *g* Supernatant	77.80 ± 8.83	71
10,000 *g* Precipitate	12·65 ± 2·93	11
10,000 *g* Supernatant	66·57 ± 2·91‡	61

* Refers to wet weight of tissue.
† Figures are means ± S.E.M.
‡ The 10,000 *g* supernatant contained an average of 0·73 mg of protein/10 mg wet weight of tissue.

shows that a linear relationship was obtained between the hydrolysis of 3′,5′-cAMP and enzyme protein between 0·3 and 1·5 mg. Figure 2 shows that a linear relationship was also obtained between enzyme activity and length of incubation time between 5 and 60 min with an enzyme preparation containing 0·7 mg of protein. Figure 3 shows the relationship between enzyme activity and substrate concentration. Detectable

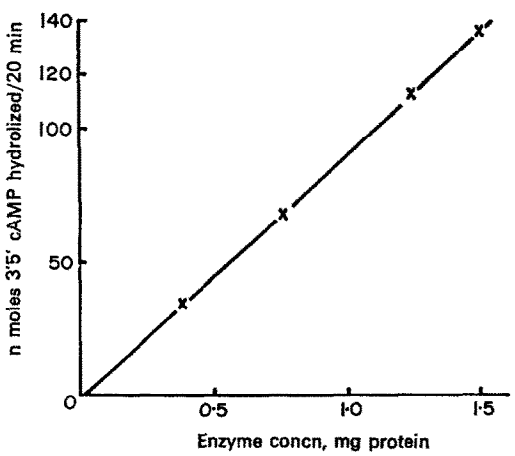

FIG. 1. Hydrolysis of 3′,5′-cAMP by various concentrations of guinea-pig lung 10,000 *g* supernatant. Each point is the average of duplicate incubations from three separate experiments. Substrate concentration was 1 mM.

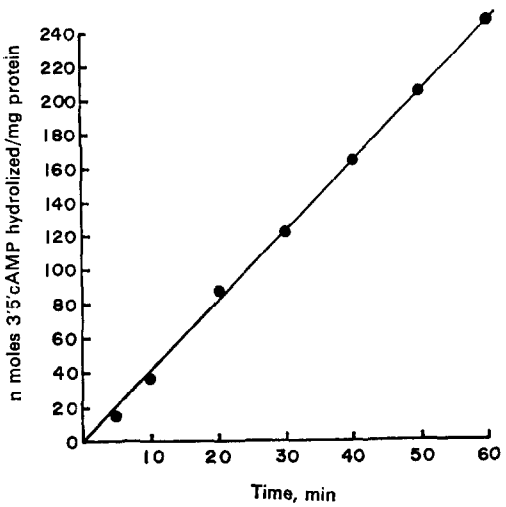

FIG. 2. Rate of hydrolysis of 3',5'-cAMP by guinea-pig lung 10,000 *g* supernatant. Each point is the average of duplicate incubations from three separate experiments. Average protein concentration was 0·7 mg/incubate. Substrate concentration was 1 mM.

amounts of 5'-AMP were formed when concentrations of 3',5'-cAMP of 0·005 mM and above were used. Maximum activity occurred at about 2·5 mM. Figure 4 (upper panel) shows a double reciprocal plot of product vs substrate concentration for the data given in Fig. 3. Guinea-pig lung 3',5'-cyclic nucleotide phosphodiesterase shows abnormal kinetics. The plot in Fig. 4 (upper panel) shows an apparent $K_m \simeq 5{\cdot}7 \times 10^{-5}$ M, and $V_{max} \simeq 35$ nmoles cAMP hydrolyzed/mg protein/20 min. A separate double reciprocal plot at the high substrate concentrations is shown in Fig. 4 (lower

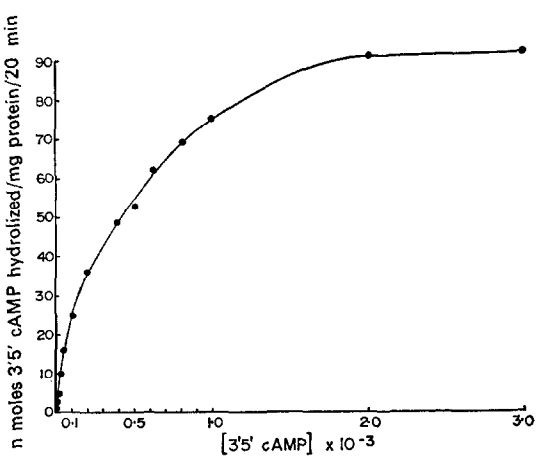

FIG. 3. Hydrolysis of 3',5'-cAMP by guinea-pig lung 10,000 *g* supernatant at different substrate concentrations. Each point is the average of duplicate incubations from three separate experiments. Average protein concentration was 0·73 mg/incubate.

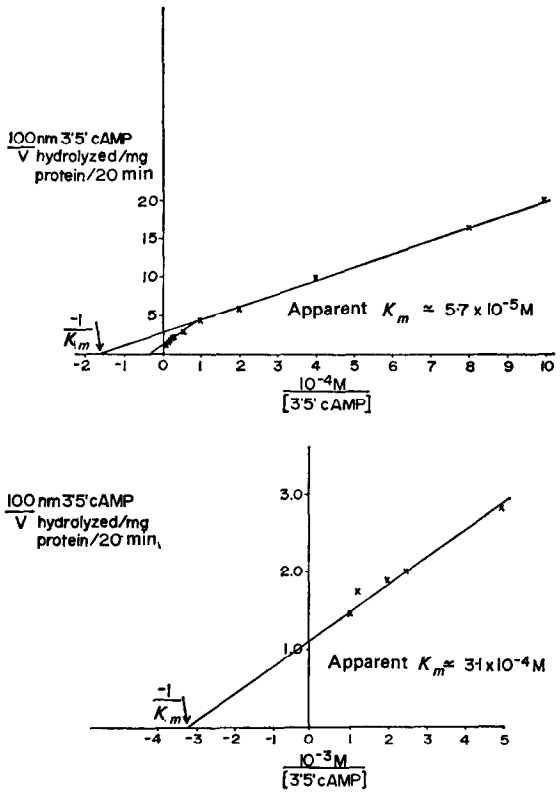

FIG. 4. Lineweaver–Burk plots of hydrolysis of 3',5'-cAMP by guinea-pig lung 10,000 *g* supernatant. The upper panel is the apparent K_m value obtained at substrate concentrations between 0·1 and 0·01 mM. The lower panel is the apparent K_m value obtained at substrate concentrations between 0·2 and 2·5 mM.

panel) and indicates the presence of a phosphodiesterase with a lower affinity for 3',5'-cAMP and an apparent $K_m \simeq 3{\cdot}1 \times 10^{-4}$ M and $V_{max} \simeq 90$ nmoles cAMP hydrolyzed/mg protein/20 min.

In all of the subsequent studies to be described, guinea-pig lung phosphodiesterase was studied at substrate concentrations of 1 and 0·01 mM. These concentrations were used to reflect predominantly the low affinity and high affinity enzyme forms respectively. However, from the K_m and V_{max} data given in Fig. 4, it was estimated that the high affinity phosphodiesterase contributed approx. 38 per cent of the total enzyme activity at 1 mM substrate concentration and that the low affinity form contributed approx. 39 per cent of the total enzyme activity at 0·01 mM substrate concentration.

Figure 5 shows the effect of some divalent cations on both low and high affinity phosphodiesterase. Magnesium which is essential for enzyme activity can be replaced by cobalt and manganese but not by copper. Under the conditions used in this study, optimum enzyme activity was obtained with 4 mM magnesium, 0·5 mM manganese and 1 mM cobalt.

Effect of methylxanthines and calcium on guinea-pig lung phosphodiesterase. Figure 6 shows the log dose–response curves obtained for the inhibition of both forms of

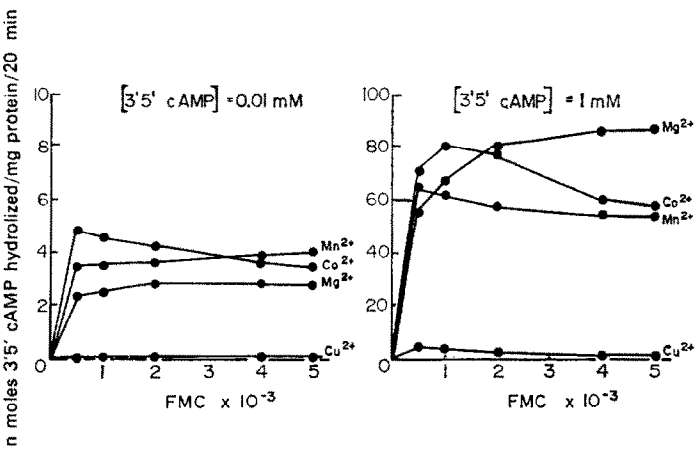

FIG. 5. Effect of divalent cations on high affinity ([S] = 0·01 mM) and low affinity ([S] = 1·0 mM) 3′,5′-cAMP phosphodiesterase in guinea-pig lung 10,000 *g* supernatant. The chloride salts of the ions were used in all cases. $MgCl_2$ was omitted from the experiments with Mn^{2+} Co^{2+} and Cu^{2+}. Each point is the average of duplicate incubations from three separate experiments. Average protein was 0·73 mg/incubate.

phosphodiesterase by aminophylline, theophylline and calcium. The methylxanthines inhibited both phosphodiesterase forms to approximately the same extent. Calcium inhibited the low affinity phosphodiesterase but had no detectable effect on the high affinity form. Under the conditions used in this study there was no stimulation of enzyme activity by low concentrations of calcium as observed by Kakiuchi and Yamasaki[9] in rat brain cortex.

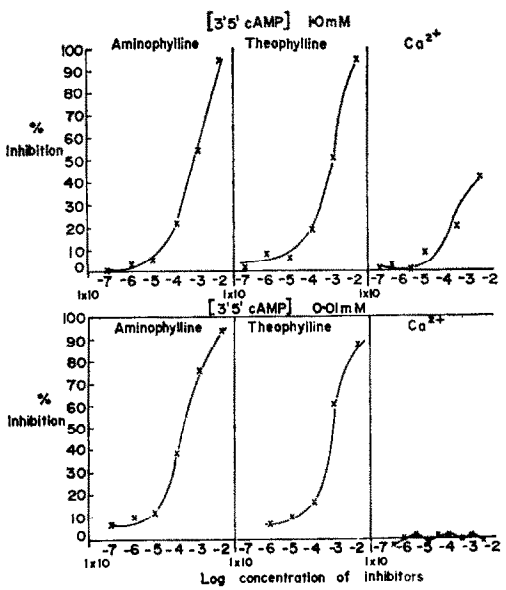

FIG. 6. Effect of aminophylline, theophylline and $CaCl_2$ on low affinity ([S] = 1·0 mM) and high affinity ([S] = 0·01 mM) 3′,5′-cAMP phosphodiesterase in guinea-pig lung 10,000 *g* supernatant. Each point is the average of duplicate incubations from three separate experiments.

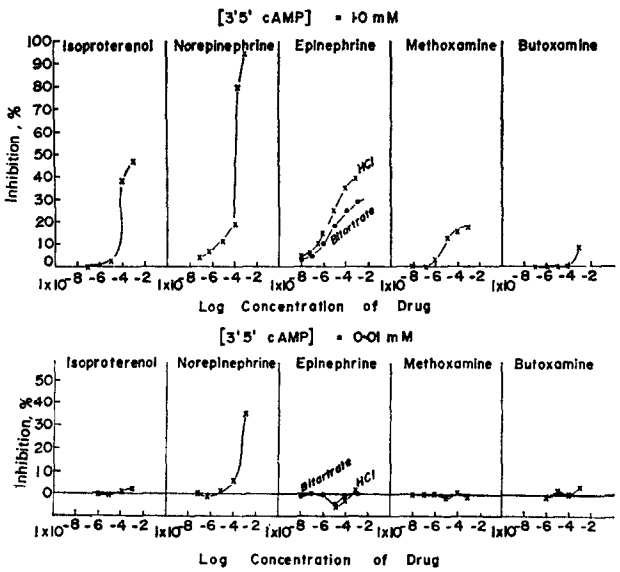

FIG. 7. Effect of adrenergic drugs on low affinity ([S] = 1·0 mM) and high affinity 3′,5′-cAMP ([S] = 0·01 mM) phosphodiesterase in guinea-pig lung 10,000 *g* supernatant. Each point is the average of duplicate incubations from three separate experiments.

Effect of adrenergic drugs on guinea-pig lung phosphodiesterase. Figure 7 shows the log dose–response curves obtained for the inhibition of both forms of phosphodiesterase by adrenergic drugs. Isoproterenol, norepinephrine and epinephrine were strong inhibitors of the low affinity enzyme. Methoxamine was a weak inhibitor and butoxamine was ineffective. Only norepinephrine inhibited the high affinity form but the degree and effective concentration range were markedly reduced as compared with its effect on the low affinity enzyme. There appeared to be a very small but consistent stimulation of the high affinity phosphodiesterase by epinephrine at 10^{-5} M.

TABLE 2. EFFECT OF MAGNESIUM ION CONCENTRATION ON THE INHIBITION OF LOW AFFINITY 3′,5′-C-AMP PHOSPHODIESTERASE BY ADRENERGIC DRUGS*

	% inhibition of control†		
$[Mg^{2+}]$	2 mM	5 mM	10 mM
Isoproterenol*	39·1 ± 1·40 (3)‡	38·6 ± 4·40 (3)‡	33·4 ± 1·76 (3)‡
Epinephrine HCl*	33·0 ± 3·64 (4)	38·4 ± 4·05 (4)	35·7 ± 1·36 (4)
Epinephrine bitartrate*	25·04 ± 2·1 (4)	23·9 ± 0·95 (4)	22·5 ± 1·43 (4)
Norepinephrine*	21·9 ± 1·85 (3)	19·0 ± 0·94 (3)	
Methoxamine*	15·0 ± 1·07 (3)	12·0 ± 0·7 (3)	16·2 ± 0·96 (4)
Control† (nmoles cAMP hydrolyzed/ mg of protein/20 min)	79·96 ± 3·55 (17)	98·54 ± 4·52 (16)	103·00 ± 6·60 (5)

* All drug concentrations were 0·1 mM.
† Concentration of 3′,5′-cAMP was 1 mM.
‡ Figures are means ± S.E.M. Figures in parentheses refer to number of experiments performed.

Since magnesium is required for phosphodiesterase activity, it was possible that the inhibition of the low activity form was due in part to chelation of the magnesium ion. Under the conditions used in this study, 2 mM magnesium gave 90 per cent of maximum activity (Fig. 5) and thus chelation of magnesium at this concentration would result in an apparent inhibition of the enzyme. Table 2 shows the degree of inhibition of the low affinity phosphodiesterase by adrenergic drugs at various concentrations of magnesium. The inhibitory effects of isoproterenol, norepinephrine, epinephrine and methoxamine appeared to be independent of the concentration of magnesium and thus chelation of the magnesium ion did not appear to be responsible for the inhibition observed.

Physiological amounts of calcium have been reported to be essential for optimum cAMP phosphodiesterase activity in rat brain.[9] Thus the inhibiting effects of the adrenergic agents could have been due to chelation of endogenous calcium ions. Addition of 10^{-4} to 10^{-6} M calcium failed to reverse the inhibition of low affinity phosphodiesterase by epinephrine hydrochloride (0·1 mM) and thus chelation of endogenous calcium did not appear to be responsible for the effect observed.

Kinetic studies on the inhibition of low affinity phosphodiesterase by epinephrine and theophylline. Figure 8 is a double reciprocal plot of enzyme activity vs substrate concentrations between 0·25 and 2·0 mM with and without epinephrine hydrochloride (0·1 mM) and theophylline (1·0 mM). Epinephrine appeared to be a noncompetitive inhibitor whereas theophylline is a competitive inhibitor of guinea-pig lung low affinity phosphodiesterase.

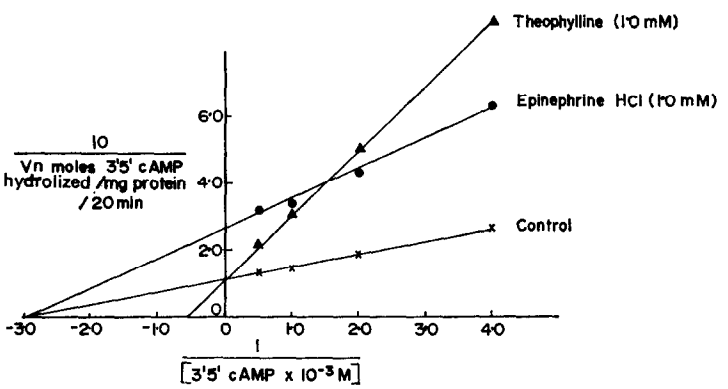

FIG. 8. Lineweaver–Burk plots of the hydrolysis of low affinity 3′,5′-cAMP phosphodiesterase in guinea-pig lung 10,000 *g* supernatant with and without theophylline and epinephrine. Average protein was 0·75 mg/incubate.

Effect of adrenergic blockers on the inhibition by epinephrine and isoproterenol. Figure 9 shows the effect of propranolol (0·1 mM) and phentolamine (0·05 mM) on the inhibition of low affinity phosphodiesterase by isoproterenol (0·1 mM) and epinephrine (0·1 mM). Propranolol and phentolamine at the concentrations used had no effect on the enzyme. Propranolol had no effect on the inhibition produced by isoproterenol and both salts of epinephrine. Thus a β-adrenergic mechanism cannot explain the inhibition by these compounds. Phentolamine had no effect on the

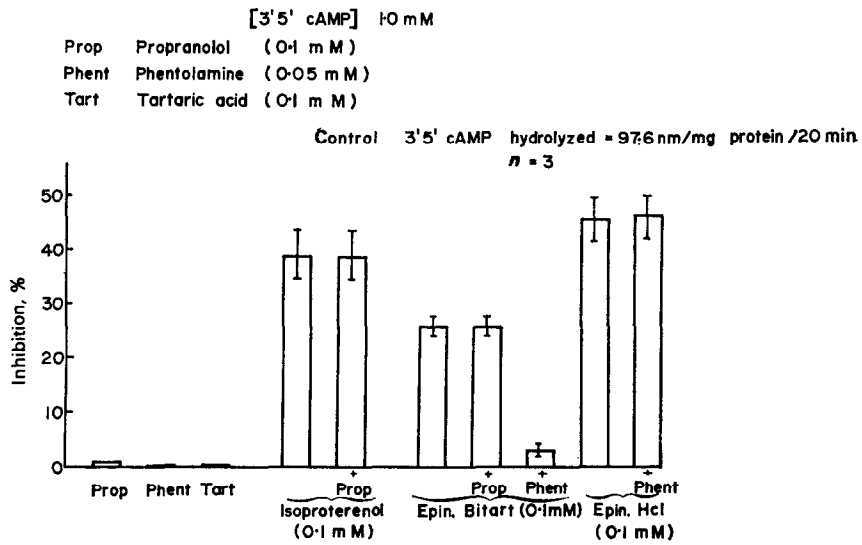

FIG. 9. Effect of adrenergic blocking agents on the inhibition of low affinity 3′,5′-cAMP phosphodiesterase in guinea-pig 10,000 *g* supernatant. Each point is the average ± S.E.M. of duplicate incubations from three separate experiments. Average protein was 0·75 mg/incubate.

inhibition produced by epinephrine hydrochloride but reversed most of that produced by epinephrine bitartrate. Separate experiments established that this was not due to the bitartrate ion (Fig. 9). The reversal of the inhibitory effect of epinephrine bitartrate occurred at phentolamine concentrations of 0·1 mM–1 μM and was independent of the magnesium ion concentration. Similar experiments with the 10,000 *g* particulate fraction produced the same results. Phenoxybenzamine (0·1 mM) reversed in part the inhibition of low affinity phosphodiesterase by both forms of epinephrine (0·1 mM). However, these experiments were complicated by the fact that 0·1 mM phenoxybenzamine alone caused about 10 per cent inhibition of low affinity phosphodiesterase and lower concentrations failed to reverse the effect of epinephrine.

DISCUSSION

This study has shown that the properties of guinea-pig lung phosphodiesterase are similar to those observed in beef heart[10] and rat brain.[7] Thus guinea-pig lung contains phosphodiesterase with different affinities for cAMP. The curved double reciprocal plot from which the two affinity constants were derived (Fig. 4) could indicate the presence of two forms of phosphodiesterase or a single enzyme displaying negative cooperativity.[11] The intracellular distribution of the low affinity form ($K_m \simeq 3{\cdot}1 \times 10^{-4}$ M) is similar to that reported by Beavo *et al.*[7] for rat brain.

The low and high affinity forms of cAMP phosphodiesterase have certain properties in common. Both require magnesium which can be replaced by manganese or cobalt but not by copper. Both forms were inhibited to approximately the same extent by methylxanthines. The degree of inhibition by 1 mM theophylline was similar to that observed for low affinity cAMP phosphodiesterase in beef heart[10] and to that observed for the high affinity form in rabbit skeletal muscle.[8]

The low and high affinity phosphodiesterases in guinea-pig lung differ markedly in their response to calcium and adrenergic drugs. Calcium inhibited the low affinity phosphodiesterase but had no effect on the high affinity form. There was no stimulation of enzyme activity by low concentrations of calcium as reported for rat brain cortex by Kakiuchi and Yamasaki,[9] and thus it must be concluded that there was sufficient endogenous calcium present in the enzyme preparation used in this study for maximum activity. Isoproterenol, epinephrine and methoxamine inhibited the low affinity phosphodiesterase but not the high affinity form. At high concentrations norepinephrine was a very potent inhibitor of low affinity phosphodiesterase and caused some inhibition of the high affinity form. The effective concentration range and the degree of inhibition of the latter enzyme was not as great when compared to its effect on the low affinity form.

A comparison of the inhibitory potencies of the adrenergic drugs used at a concentration of 0·1 mM shows that isoproterenol and epinephrine hydrochloride were equipotent and more effective than norepinephrine and methoxamine. A comparison of the shape of the log dose–response curves for the inhibition of low affinity phosphodiesterase by all of the drugs employed shows that they can be separated into two groups. Isoproterenol, epinephrine, methoxamine and calcium have dose–response curves which are similar in that the inhibitory effect is spread over a wide range of concentration and that maximum inhibition did not exceed 50 per cent. The shape of the log dose–response curves for norepinephrine, aminophylline and theophylline is similar in that they are very steep, and that 95 per cent inhibition of enzyme activity could be obtained at high concentrations. Moreover, this group of compounds are the only drugs studied which were effective inhibitors of the high affinity enzyme.

Kinetic studies showed that the nature of the inhibition of phosphodiesterase by theophylline and epinephrine is different. Theophylline is a competitive inhibitor of low affinity phosphodiesterase since it raises the K_m without effecting the V_{max}. The studies of Huang and Kemp[8] showed that theophylline exhibits similar kinetic behavior with high affinity phosphodiesterase. It should be pointed out that since the low affinity form contributed approx. 39 per cent to the overall enzyme activity at 0·01 mM substrate concentration, an agent which could raise the K_m of the high K_m form without affecting its V_{max} would appear to selectively inhibit the low K_m form. The agents used in this study did not appear to show such behavior. Epinephrine hydrochloride was a noncompetitive inhibitor of low affinity phosphodiesterase since it had no effect on the K_m but decreased the V_{max}. Similar studies with epinephrine bitartrate did not provide clear-cut results. Goren and Rosen[12] recently reported that epinephrine is a noncompetitive inhibitor of beef heart phosphodiesterase. The experimental conditions of their investigation suggest that they were also studying the low affinity enzyme. The data from this study as well as those of Goren and Rosen[12] contrast markedly with the observation of Amer[5] that epinephrine lowered the K_m of low affinity phosphodiesterase in rat liver.

The mechanism of the inhibition of the low affinity phosphodiesterase produced inconclusive results. Chelation of either magnesium or endogenous calcium which are known to be essential for optimal enzyme activity did not appear to be responsible for the inhibitory effect of epinephrine. Propranolol failed to reverse the inhibitory effect of both epinephrine and isoproterenol, suggesting that a β-adrenergic mechanism was not operating under the experimental conditions used. Phentolamine blocked

almost all of the inhibitory effect of epinephrine bitartrate but had no effect on the inhibition produced by epinephrine hydrochloride. The selective reversal by phentolamine of the inhibitory effect of one form of epinephrine but not the other cannot be explained at this time. Goren and Rosen[12] were unable to block the inhibitory effects of epinephrine on beef heart phosphodiesterase although they did not specify which form of epinephrine was used.

Intracellular levels of cAMP in chopped human lung fragments have been shown to increase after adrenergic stimulation with isoproterenol, epinephrine and norepinephrine. The physiological effect was an inhibition of the immunological release of histamine within 5 min. Aminophylline also produced the same effects over the same time period, clearly via inhibition of phosphodiesterase. The similarity of effects of the two types of drugs suggests that the increased cAMP levels observed after adrenergic stimulation might have been due in part to phosphodiesterase inhibition. However, on the basis of the data presented in this report it must be concluded that there is insufficient evidence to support either an α- or β-adrenergic receptor mechanism for the inhibition of low affinity phosphodiesterase by epinephrine.

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