Biochemical Pharmacology, Vol. 26, pp. 951–954. Pergamon Press, 1977. Printed in Great Britain.

EFFECT OF SCH 15280, A NEW BRONCHODILATOR, ON CYCLIC 3′,5′-NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY AND CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE LEVELS IN GUINEA PIG LUNG

WILLIAM KREUTNER and JOSEPH E. SHERWOOD
Department of Molecular Biology, Schering Corporation, Bloomfield, NJ 07003, U.S.A.

(*Received* 8 *July* 1976; *accepted* 22 *October* 1976)

Abstract—Sch 15280, 5[4-(*N*-methyl)-piperidylidene]5H-(1)benzopyrano(2,3,b)pyridine maleate is a bronchodilator and antihistamine in animal models. The bronchodilatation produced by Sch 15280 and aminophylline are not antagonized by β-receptor blockade. Sch 15280 and aminophylline inhibited the high affinity cyclic AMP phosphodiesterase of guinea pig whole lung and bronchi. Sch 15280 was approximately one-half as potent as aminophylline. Aminophylline increased the concentration of cyclic AMP and potentiated the cyclic nucleotide elevation due to isoproterenol in lung tissue *in vitro*. Neither effect was elicited by Sch 15280. Burimamide (anti-H_2) and chlorpheniramine (anti-H_1) antagonized the cyclic AMP elevation caused by histamine. Sch 15280 (anti-H_1) failed to alter the response to histamine. The bronchodilatory activity of Sch 15280 may be independent of its inhibitory effects on cyclic AMP phosphodiesterase activity.

Sch 15280, 5[4-(*N*-methyl)-piperidylidene]5H-(1)benzopyrano(2,3,b)pyridine maleate, has shown bronchodilator and antihistaminic properties in animal models [1, 2]. In addition, this compound has been shown to inhibit antigen-induced release of histamine from sensitized rat mast cells [3].

Numerous investigations have indicated a role for intracellular cyclic 3′,5′-adenosine monophosphate (cyclic AMP) in the regulation of smooth muscle tone and antigen-induced mediator release from sensitized tissue [4–6]. Stimulation of β-adrenergic receptors by agents such as isoproterenol and the resultant bronchodilatation has been associated with an activation of membrane bound adenylate cyclase and increased synthesis of cyclic AMP [7, 8].

The tissue level of cyclic AMP is also controlled by its rate of degradation by cyclic AMP phosphodiesterases (E.C.3.1.4.c) [9]. The intrinsic bronchodilator activity of the methyl xanthines (e.g. aminophylline), as well as their ability to potentiate the action of catecholamines, is due in large measure to the inhibition of cyclic AMP phosphodiesterase activity by these compounds [5–7].

The bronchodilatation produced by Sch 15280 and the methyl xanthines are not sensitive to β-adrenergic receptor blockade. We therefore investigated the effect of Sch 15280 on cyclic AMP phosphodiesterase activity of guinea pig lung and compared it to that of aminophylline. In addition, the effects of these compounds on basal and hormone-induced cyclic AMP levels were measured in guinea pig lung slices *in vitro*.

MATERIALS AND METHODS

Assay of cyclic 3′,5′-nucleotide phosphodiesterase. Cyclic 3′,5′-nucleotide phosphodiesterase activity was determined by the radiometric method of Brooker *et al.* [10] on the 70,000 *g* supernatant obtained from the whole lungs or dissected bronchi of male albino guinea pigs (Marland Farms, 300 g body wt). Previous studies have emphasized the importance in using a low concentration of substrate to assay the activity of the high affinity form of phosphodiesterase [9]. Therefore, the substrate used for inhibition studies was either 1 μM cyclic AMP or 1 μM cyclic GMP. In other kinetic studies the concentration of cyclic AMP was varied between 0.1–1 μM to determine the nature of the inhibition and the K_i of the inhibitor. Preliminary experiments established that reaction velocity was linear with respect to time and enzyme concentration. The test compounds were added to the assay solution containing enzyme 5 min before initiating the reaction with the addition of substrate. All assays were performed in triplicate.

Effect of compounds on cyclic AMP levels. Male albino guinea pigs (Marland Farms, 300 g body wt) were killed by decapitation and the lungs quickly removed and cleaned of visible connective tissue and blood vessels. In each experiment, the lungs from 3–5 guinea pigs were pooled, sliced into 1 mm cubes and suspended in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 1 mg/ml glucose. One milliliter aliquots, containing approximately 15 mg of tissue protein were transferred to incubation vials. The tissues were incubated for 5 min at 37° in an atmosphere of 95% O_2: 5% CO_2 in the presence or absence of various compounds, after which either histamine or isoproterenol were added where indicated, and the incubation was continued an additional 5 min. The reaction was terminated by adding 0.2 ml of 36% trichloroacetic acid. Extracts from the incubation mixture (tissue plus medium) were washed with acidified diethyl ether and the cyclic AMP content of the aqueous phase determined by the protein binding

method of Gilman [11]. Standard concentrations of cyclic AMP were prepared in Krebs–Ringer bicarbonate buffer (in the absence of tissue) and carried through the entire procedure. None of the agents tested interfered with the assay for cyclic AMP.

The trichloracetic acid precipitable protein content of the tissues were assayed by the method of Lowry *et al.* [12] using bovine serum albumin as a standard. The levels of cyclic AMP were expressed as pmoles per mg of protein. Statistical comparisons were made using Student's *t*-test for paired comparisons.

Aminophylline, histamine dihydrochloride, and DL-propranolol-HCl were obtained from Sigma Chemical Co. Burimamide was kindly supplied by Smith Kline and French Laboratories. DL-Isoproterenol-HCl was obtained from Ayerst Laboratories. [^{3}H]Cyclic AMP (24 Ci/m-mole) and [^{3}H]cyclic GMP (2.1 Ci/m-mole) were purchased from New England Nuclear. DL-Chlorpheniramine-maleate and Sch 15280 were synthesized in the Schering Laboratories department of Medicinal Chemistry.

RESULTS

Inhibition of cyclic 3',5'-nucleotide phosphodiesterase activity. Table 1 summarizes the inhibition of lung and bronchi cyclic AMP phosphodiesterase activity produced by aminophylline and Sch 15280 at a cyclic AMP concentration of 1 μM. From the IC_{50} values it can be seen that Sch 15280 is approximately one-half as potent an inhibitor as aminophylline. Furthermore, both compounds inhibited the hydrolysis of cyclic GMP with IC_{50} values similar to those against cyclic AMP phosphodiesterase.

The kinetics of the hydrolysis of cyclic AMP by the guinea pig lung phosphodiesterase and the inhibition by aminophylline and Sch 15280 were investigated by Lineweaver–Burk analysis and are presented in Fig. 1. The K_m for hydrolysis of cyclic AMP in the absence of inhibitor was 9×10^{-7} M. The inhibition produced by aminophylline appeared competitive with a K_i of 1.2×10^{-4} M. In contrast, the inhibition produced by Sch 15280 appeared non-competitive and the K_i was calculated to be 2.5×10^{-4} M. The kinetics of the hydrolysis of cyclic GMP was not examined.

Cyclic AMP levels in lung slices. In all experiments cyclic AMP was determined in the entire incubation mixture, tissue plus medium. Studies by Murad and Kimura [6] and Lindl and Cramer [13] have shown that significant quantities of cyclic AMP are released into the medium during incubation in the absence or presence of hormones and that total (tissue plus medium) levels of cyclic AMP adequately reflect changes in tissue cyclic AMP.

Table 1. IC_{50} of phosphodiesterase inhibitors*

Drug	Cyclic AMP Phosphodiesterase Lung	Cyclic AMP Phosphodiesterase Bronchi	Cyclic GMP Phosphodiesterase Lung
Aminophylline	0.13	0.14	0.16
Sch 15280	0.28	0.39	0.37

* IC_{50} = Millimolar concentration causing 50 per cent inhibition at a substrate concentration of 1 μM cyclic AMP or 1 μM cyclic GMP. Values were calculated from the data in Fig. 1.

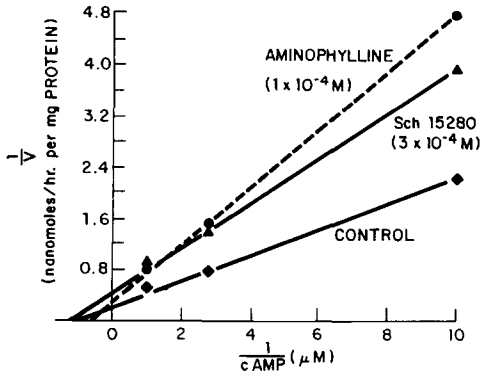

Fig. 1. Double-reciprocal plot of the hydrolysis of cyclic AMP by the low K_m cyclic AMP phosphodiesterase and the inhibition by aminophylline and Sch 15280. Each point represents the mean of two experiments.

Incubation of lung slices for 10 min with 10^{-3} M aminophylline produced a significant accumulation of cyclic AMP whereas Sch 15280 (10^{-4} and 10^{-3} M) was inactive (Table 2). Also, aminophylline (10^{-3} M) but not Sch 15280 increased the amount of cyclic AMP accumulated during a 5 min exposure to 10^{-5} M isoproterenol.

The effect of phosphodiesterase inhibitors and antihistamines on the cyclic AMP accumulation produced by histamine (5×10^{-5} M) are shown in Table 3. Aminophylline markedly increased the level of cyclic AMP above that seen with histamine alone. The response to histamine was not altered in the presence of 10^{-4} M Sch 15280 but was significantly reduced by 10^{-4} M of the antihistamine burimamide (anti-H_2) or chlorpheniramine (anti-H_1). Burimamide appeared somewhat more effective than chlorpheniramine at the concentration tested. Other experiments (not shown) confirmed that chlorpheniramine and burimamide did not alter basal cyclic AMP levels in our lung preparation.

Table 2. Effects of phosphodiesterase inhibitors in the absence and in the presence of isoproterenol on cyclic AMP levels in lung

Addition	Cyclic AMP/(pmole/mg protein) Basal	Isoproterenol (10^{-5} M)
None	36 ± 6	73 ± 8*
Aminophylline (10^{-4} M)	41 ± 6	100 ± 18
Aminophylline (10^{-3} M)	116 ± 12*	206 ± 26†
Sch 15280 (10^{-4} M)	34 ± 5	87 ± 10
Sch 15280 (10^{-3} M)	40 ± 5	82 ± 5

Lung slices were incubated for 5 min in the absence or presence of either aminophylline or Sch 15280. Isoproterenol was then added where indicated and the incubation continued an additional 5 min. Cyclic AMP levels in tissue plus medium are reported as the mean ± S.E.M. of 6 experiments.

* $P < 0.01$ isoproterenol or phosphodiesterase inhibitors compared to basal incubation (no additions).

† $P < 0.01$ compared to isoproterenol alone.

Table 3. Effect of agents on histamine-induced cyclic AMP levels in lung

Addition	Cyclic AMP (pmole/mg protein)
Series I	
None	36 ± 5
Histamine (H)	135 ± 18*
(H) + Aminophylline (10^{-3} M)	333 ± 24†
(H) + Sch 15280 (10^{-4} M)	122 ± 14
Series II	
None	32 ± 3
Histamine (H)	160 ± 7*
(H) + Burimamide (10^{-4} M)	56 ± 6†
(H) + Chlorpheniramine (10^{-4} M)	82 ± 9‡

Lung slices were incubated for 5 min in the absence or presence of either aminophylline, Sch 15280, burimamide, or chlorpheniramine. Histamine (5×10^{-5} M final concentration) was then added where indicated and the incubation continued an additional 5 min. Cyclic AMP levels in tissue plus medium are reported as the mean ± S.E.M. of 6 experiments.

* $P < 0.01$ histamine alone compared to basal incubation (no addition).

† $P < 0.01$ compared to histamine alone.

‡ $P < 0.05$ compared to histamine alone.

DISCUSSION

It is established that hormonal stimulation of bronchodilatation is secondary to an increase in cyclic AMP levels. Cyclic AMP phosphodiesterases represent a possible target for the pharmacological modulation of the intracellular concentration of cyclic AMP. The methyl xanthines are known to produce both phosphodiesterase inhibition and bronchodilatation. Sch 15280 is a new, non-adrenergic, bronchodilator which has been shown to relax the isolated guinea pig trachea, delay histamine-induced dyspnea, decrease pulmonary resistance and increase compliance in dogs and rats [1], inhibit methacholine-induced bronchoconstriction [2], and inhibit antigen-induced histamine release from sensitized rat mast cells [3]. Sch 15280 was approximately one-half the potency of aminophylline as an inhibitor of the low K_m cyclic AMP phosphodiesterase of guinea pig lung and bronchi. Unlike aminophylline, however, Sch 15280 did not increase the concentration of cyclic AMP nor potentiate the cyclic nucleotide elevation due to isoproterenol in lung tissue *in vitro*.

The lack of a significant increase in lung cyclic AMP levels with Sch 15280 may indicate that the compound does not penetrate the intact cell membrane for exposure to intracellular cyclic AMP phosphodiesterase and suggests that its bronchodilatory activity may be independent of its inhibitory effects on cytosolic cyclic AMP phosphodiesterase activity. It is possible, however, that because of its heterogeneous nature our lung tissue may not reflect cyclic AMP responses of the smooth muscle or mast cell components to phosphodiesterase inhibition.

It is often difficult to correlate the activity of agents as inhibitors of cyclic AMP phosphodiesterase with their pharmacological action. Polacek *et al.* [14] demonstrated that theophylline relaxed the rat uteri with no change in cyclic AMP concentration. Neither papaverine [15] nor SQ 20009 [16] raised the levels of cyclic AMP or stimulated lipolysis of intact adipocytes despite potent inhibition of cyclic AMP phosphodiesterase activity in cell homogenates.

Activation of H_2-receptors by histamine raises the tissue concentration of cyclic AMP [4, 6, 7, 13, 17–19]. This effect is antagonized by burimamide (anti-H_2) and in some cases by classical antagonists of H_1-responses [6, 7, 13, 19]. Other workers have reported this effect of histamine was not blocked by H_1-antagonists [4, 17, 18]. McNeil and Verma [20] reported that antagonism of the histamine (H_2) response on the heart by promethazine (anti-H_1) was not of a competitive nature.

The failure of Sch 15280 (anti-H_1) to attenuate the cyclic AMP response to histamine is surprising since chlorpheniramine (anti-H_1) significantly diminished the histamine effect. The possibility exists that at the relatively high concentrations used in these experiments, anti-H_1 agents such as chlorpheniramine also antagonize H_2-responses whereas Sch 15280 exhibits less affinity for the H_2-receptor than does chlorpheniramine.

The mechanism by which Sch 15280 causes bronchodilatation and inhibition of mast cell histamine release remains unknown. Cyclic 3′,5′-guanosine monophosphate (cyclic GMP) and cyclic AMP are antagonistic physiological regulators of cell function [21]. Elevation of cellular cyclic GMP levels has been associated with respiratory smooth muscle contraction [8] and immunologic release of histamine [4, 5]. The effect of Sch 15280 on immunologic and cholinergic-induced increases in lung cyclic GMP remains to be studied.

The interaction between Sch 15280 and calcium (Ca^{2+}) transport may also shed light on the nature of the bronchodilatory action. Ca^{2+} has an integral role in smooth muscle contraction and histamine release [22]. A change in Ca^{2+} transport or cellular localization could cause bronchial relaxation [14]. Also, it is evident that Ca^{2+} modulates the adenylate cyclase and phosphodiesterase systems responsible for controlling the cellular concentration of cyclic AMP [23].

Acknowledgement—The authors wish to thank Mrs. Suzanne Springer for excellent technical assistance in the performance of these experiments.

REFERENCES

1. S. Tozzi, *Fedn Proc.* **33**, 569, (1974).
2. L. Diamond, G. K. Adams, B. Bleidt and B. Williams, *J. Pharmac. exp. Ther.* **193**, 256, (1975).
3. S. Smith, S. Tozzi and J. Petillo, *Abstracts of the 30th Annual Meeting of the American Academy of Allergy*, p. 25, (1974).
4. L. M. Lichtenstein and E. Gillespie, *Fedn Proc.* **32**, 1010, (1973).
5. M. Kaliner and K. F. Austen, *Biochem. Pharmac.* **23**, 763, (1974).
6. F. Murad and H. Kimura, *Biochim. biophys. Acta* **343**, 275, (1974).
7. G. C. Palmer, *Biochem. Pharmac.* **21**, 2907, (1972).
8. J. F. Kuo and W. Kuo, *Biochem. biophys. Res. Commun.* **55**, 660, (1973).
9. M. S. Amer and W. E. Kreighbaum, *J. pharm. Sci.* **64**, 1, (1975).

10. G. Brooker, L. J. Thomas and M. M. Appleman, *Biochemistry* **7,** 4177, (1968).
11. A. G. Gilman, *Proc. natn. Acad. Sci. U.S.A.* **67,** 305, (1970).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265, (1951).
13. T. Lindl and H. Cramer, *Biochim. biophys. Acta* **343,** 182, (1974).
14. I. Polacek, J. Bolan and E. E. Daniel, *Can. J. Physiol. Pharmac.* **49,** 999, (1971).
15. J. A. Beavo, N. L. Rogers, O. B. Crofford, C. E. Baird, J. G. Hardman, E. W. Sutherland and E. V. Newman, *Ann. N.Y. Acad. Sci.* **185,** 129, (1971).
16. D. O. Allen, J. F. Clark and J. Ashmore, *J. Pharmac. exp. Ther.* **185,** 379, (1973).
17. G. C. Palmer, *Biochim. biophys. Acta* **252,** 561, (1971).
18. L. M. Lichtenstein and E. Gillespie, *J. Pharmac. exp. Ther.* **192,** 441, (1975).
19. I. Weinryb and I. M. Michel, *J. med. Chem.* **18,** 23, (1975).
20. J. H. McNeill and S. C. Verma, *Can. J. Physiol. Pharmac.* **52,** 23, (1974).
21. N. D. Goldberg, R. F. O'Dea and M. K. Haddox, in *Advances in Cyclic Nucleotide Research* (Ed. P. Greengard and G. A. Robison), vol. 3, p. 155, Raven Press, N.Y. (1973).
22. L. M. Lichtenstein, *Int. Archs Allergy appl. Immun.* **49,** 143, (1975).
23. F. Carpendo, R. M. Gaion and G. Fassina, *Biochem. Pharmac.* **24,** 2069, (1975).