Biochemical Pharmacology, Vol. 36, No. 2, pp. 277–281, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

EFFECTS OF THIAZINAMIUM CHLORIDE AND OTHER ANTIHISTAMINES ON PHOSPHATIDYLCHOLINE SECRETION IN RAT TYPE II PNEUMOCYTE CULTURES

ALASDAIR M. GILFILLAN,* ALAN J. LEWIS† and SEAMUS A. ROONEY*‡

*Division of Perinatal Medicine, Department of Pediatrics, Yale University School of Medicine, New Haven, CT 06510; and †Wyeth Laboratories Inc., Philadelphia, PA 19101, U.S.A.

(*Received* 10 *April* 1986; *accepted* 2 *July* 1986)

Abstract—Thiazinamium chloride (TCl) stimulated phosphatidylcholine secretion in cultures of adult rat type II pneumocytes in a concentration-dependent manner in the range 10^{-9}–10^{-6} M. At the optimal concentration, secretion was stimulated by 46% which is approximately half the stimulatory effect of the β-agonists terbutaline and isoproterenol. TCl did not increase the rate of choline incorporation into cellular phosphatidylcholine or of lactate dehydrogenase release so its effect on secretion was not secondary to phosphatidylcholine synthesis or cell injury. Since TCl has antihistaminic properties, we examined the effects of other antihistamines. The H-1 antagonists promethazine, which is structurally similar to thiazinamium, and pyrilamine, which has a different structure, also stimulated secretion but the H-2 antagonist, cimetidine, did not. The effects of TCl and pyrilamine were additive to those of terbutaline, suggesting that the mechanisms of action of the antihistamines and the β-agonist were different. Although we were unable to demonstrate an inhibitory effect of histamine itself on either basal or terbutaline-stimulated phosphatidylcholine secretion, it is possible that histamine plays a regulatory role in lung surfactant secretion.

Thiazinamium chloride (TCl), which is a quaternary analog of promethazine, is under clinical investigation as an aerosol bronchodilator drug. When administered as an aerosol to animals, it has antihistaminic and anticholinergic bronchodilator properties [1]. It also inhibits thromboxane synthesis in rat alveolar macrophages [2]. Because of its action in the lung we examined the effect of TCl on pulmonary surfactant synthesis and secretion.

Lung surfactant is phospholipid-rich material which lines the alveolar surface where it is believed to maintain alveolar stability at low lung volumes [3, 4]. Surfactant is synthesized in the alveolar type II pneumocyte, stored in lamellar inclusion bodies and secreted by exocytosis [3]. Phosphatidylcholine accounts for over 80% of surfactant phospholipids [5], and its disaturated species is largely responsible for the surface tension-lowering properties of surfactant [4]. Secretion of phosphatidylcholine by purified cultures of isolated type II pneumocytes has been used as a model in which to study regulation of surfactant secretion *in vivo* [6–9]. In this system phosphatidylcholine secretion has been reported to be stimulated by a variety of pharmacological agents including β-adrenergic agonists [6, 7], leukotrienes [9, 10], purinoceptor agonists [11] and compounds which activate protein kinase C [12]. There is evidence that some of these agents may be involved in the physiological regulation of surfactant secretion [3, 4].

In this study we examined the effect of TCl on phosphatidylcholine secretion in primary cultures of type II pneumocytes from adult rat lung. We also examined its effect on the rate of choline incorporation into phosphatidylcholine as a measure of surfactant synthesis. Some of this work has been published as an abstract [13].

‡ Correspondence should be sent to: Dr. Seamus A. Rooney, Yale Department of Pediatrics, P.O. Box 3333, New Haven, CT 06510, U.S.A.

MATERIALS AND METHODS

Type II pneumocytes were isolated from the lungs of adult male Sprague–Dawley rats (approx. 175 g) by a modification of the procedure of Brown and Longmore [6] as described previously [5]. This method involves purification of the cells by passage through a discontinuous albumin density gradient and preferential attachment on plastic culture dishes.

For secretion studies, the freshly isolated cells were plated at a density of 3.5×10^6 cells per dish and cultured for 18–20 hr in 1.5 ml of Dulbecco's modified Eagle's medium containing [*methyl*-^{3}H]choline chloride (2 μCi/ml), 10% fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100 units/ml). The cells were rinsed with fresh serum- and antibiotic-free medium to remove [^{3}H]choline and unattached cells. At this stage approximately 1×10^6 cells remained attached of which at least 95% were type II cells. After a 30-min preincubation in the fresh medium the test agents were added, and the incubation was continued for 90 min. The medium was then aspirated, the cells were lysed with ice-cold water, and lipids were extracted from both cells and medium with chloroform and methanol [5]. Phosphatidylcholine was separated from the other phospholipids by thin-layer chromatography [10], and its radioactivity was measured by liquid scintillation counting [9]. Secretion was expressed as the amount of [^{3}H]phosphatidylcholine in the medium after the 2-hr incubation as a percentage of that in the cells plus

medium. In fifteen control experiments, the amounts of radioactivity in the medium and cells were 631 ± 118 and 78,060 ± 9,960 cpm respectively.

The rate of [*methyl*-^{3}H]choline incorporation into phosphatidylcholine was measured to assess effects on phosphatidylcholine synthesis. After the 18–20 hr culture period the cells were incubated with [*methyl*-^{3}H]choline in the presence or absence of the test compounds, and cellular [^{3}H]phosphatidylcholine was measured as described previously [10, 14]. Although the rate of incorporation of a radiolabeled precursor into a product does not necessarily reflect the true rate of synthesis, the rate of choline incorporation into phosphatidylcholine has been shown to correlate well with other parameters of phosphatidylcholine synthesis in a number of lung systems [15]. To assess cellular integrity, the activity of lactate dehydrogenase (EC 1.1.1.27) in the cells and medium was measured [9] after exposure to the test compounds.

Rats were purchased from Charles River Breeding Laboratories, Kingston, NY. Tissue culture medium was from the Grand Island Biological Co., Grand Island, NY; fetal bovine serum from HyClone Laboratories, Logan, UT; and [*methyl*-^{3}H]choline from NEN Research Products, E.I. du Pont de Nemours & Co., Boston, MA. TCl and promethazine were from Wyeth Laboratories, Philadelphia, PA; terbutaline sulfate (Brethine) was from Geigy Pharmaceuticals, Ardsley, NY; and other agents and biochemicals were from the Sigma Chemical Co., St. Louis, MO.

RESULTS

The effect of TCl on phosphatidylcholine secretion in type II cells is shown in Fig. 1. TCl stimulated phosphatidylcholine secretion, and this effect was dependent on concentration in the range 10^{-8}–10^{-6} M. In the same system phosphatidylcholine secretion was also stimulated by the β-adrenergic agonists isoproterenol and terbutaline in the ranges 10^{-10}–10^{-7} M and 10^{-9}–10^{-5} M respectively (Fig. 2). However, while optimum concentrations of isoproterenol and terbutaline doubled the rate of phosphatidylcholine secretion, TCl only increased it 46% (Table 1). The EC_{50} values for TCl, isoproterenol and terbutaline were 2.9×10^{-8} M, 3.2×10^{-9} M and 1.6×10^{-7} M respectively.

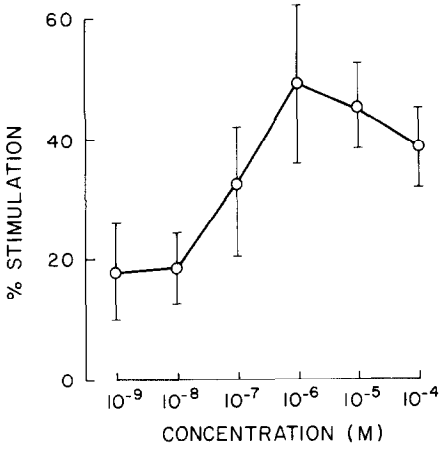

Fig. 1. Effect of TCl on phosphatidylcholine secretion in type II pneumocytes. The cells were incubated with the indicated concentration of TCl for 90 min after which phosphatidylcholine secretion was measured as described in Materials and Methods. Stimulation is expressed as the percentage increase over the rate in the absence of TCl as indicated in Table 1. The data are means ± SE (bar) from six experiments. In fifteen control experiments, the amounts of radioactivity in the medium and cells were 631 ± 118 and 78,060 ± 9,960 cpm respectively.

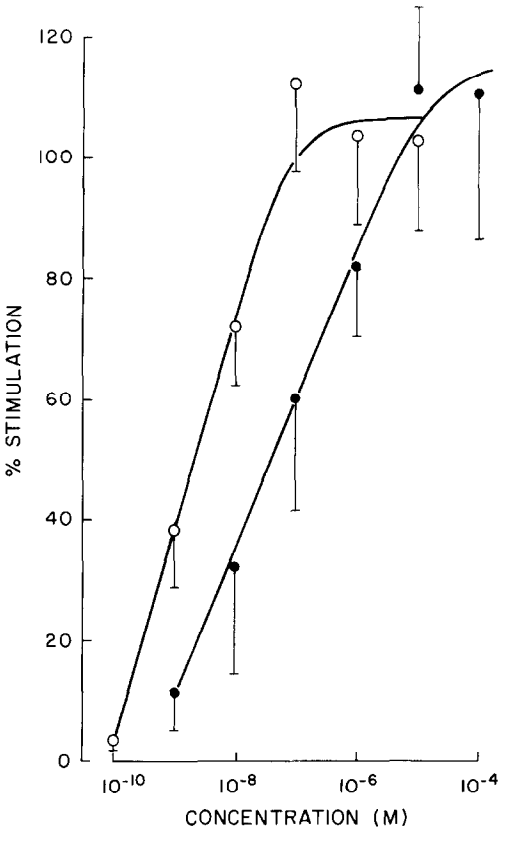

Fig. 2. Effects of various concentrations of isoproterenol (○) and terbutaline (●) on phosphatidylcholine secretion in type II cells. The isoproterenol data are from five and the terbutaline data from five to ten experiments. Other details are as in Fig. 1.

Rates of choline incorporation into cellular phosphatidylcholine and lactate dehydrogenase release were the same as reported previously [10] and were not affected by TCl. The stimulatory effect on phosphatidylcholine secretion is therefore likely to be a direct effect rather than one secondary to synthesis or cellular injury. At a higher concentration (10^{-4} M) TCl inhibited the rate of choline incorporation into phosphatidylcholine by 64% from 3200 ± 540 cpm/10^6 cells/hr in the controls to 1150 ± 220 ($N = 7$, $P < 0.002$) but had no effect on lactate dehydrogenase release.

To examine the possibility that the stimulatory effect of TCl on phosphatidylcholine secretion was due to its antihistamine action we examined the effect of two other antihistamines: promethazine

Table 1. Effects of TCl, β-agonists, antihistamines and histamine on phosphatidylcholine secretion in cultured type II pneumocytes*

Agonist (M)	N	% Phosphatidylcholine secreted		Treated/ control	P
		Control	Treated		
TCl, 10^{-6}	6	0.92 ± 0.05	1.34 ± 0.08	1.46	< 0.01
Isoproterenol, 10^{-7}	5	0.94 ± 0.19	1.95 ± 0.38	2.07	< 0.01
Terbutaline, 10^{-5}	10	0.98 ± 0.08	2.01 ± 0.13	2.05	< 0.001
Pyrilamine, 10^{-5}	7	0.58 ± 0.04	0.88 ± 0.04	1.52	< 0.002
Promethazine, 10^{-6}	7	0.83 ± 0.09	1.23 ± 0.17	1.48	< 0.01
Cimetidine, 10^{-5}	4	0.87 ± 0.08	1.02 ± 0.10	1.17	NS
Histamine, 10^{-5}	4	0.87 ± 0.08	0.90 ± 0.04	1.03	NS

* The cells were incubated with or without (controls) the indicated concentration of agonist for 90 min after which phosphatidylcholine secretion was measured as described in Materials and Methods. The data are expressed as [^{3}H]phosphatidylcholine in the medium as percentage of total (cells + medium) and are means ± SE from the number of experiments indicated (N). Statistical analysis was with Student's two-tailed *t*-test for paired samples. NS, not significant ($P > 0.05$). In fifteen control experiments, the amounts of radioactivity in the medium and cells were 631 ± 118 and 78,060 ± 9,960 cpm respectively.

which is a structural analog of thiazinamium and pyrilamine which is structurally different. As shown in Fig. 3, both of these agents stimulated phosphatidylcholine secretion in a concentration-dependent manner. The stimulatory effects of these antihistamines were similar to that of TCl (Table 1). The EC_{50} of pyrilamine was 5.6×10^{-9} M. It was therefore an order of magnitude more potent than TCl. Promethazine appeared to have potency similar to TCl although its EC_{50} was not calculated because of toxicity at the higher concentrations. In contrast to these H-1 antagonists, the H-2 antagonist cimetidine did not stimulate phosphatidylcholine secretion at concentrations of 10^{-9}–10^{-5} M (Table 1).

We examined the effects of TCl and pyrilamine in combination with terbutaline. As shown in Table 2, the combination was additive, suggesting that these agents and β-adrenergic agonists stimulate phosphatidylcholine secretion via different mechanisms.

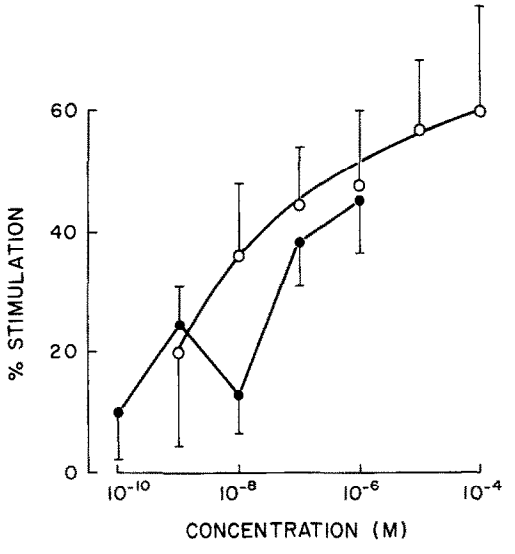

Fig. 3. Effects of various concentrations of pyrilamine (○) and promethazine (●) on phosphatidylcholine secretion in type II cells. The data are from five to seven experiments. Other details are as in Fig. 1.

Table 2. Effects of pyrilamine and TCl, alone and in combination with terbutaline, on phosphatidylcholine secretion*

Agonists	% Stimulation
Terbutaline	125 ± 15
Pyrilamine	35 ± 6
TCl	40 ± 14
Terbutaline + pyrilamine	200 ± 26
Terbutaline + TCl	161 ± 27

* Maximal concentrations of pyrilamine (10^{-5} M) or TCl (10^{-6} M) were added to the culture medium immediately after washing the cells. After 30 min, terbutaline (10^{-5} M) was added and the incubation was continued for 90 min. [^{3}H]Phosphatidylcholine secretion was then measured as described in Materials and Methods. The data are means ± SE from six pyrilamine and five TCl experiments. Control values are in the legend of Table 1. Stimulation is expressed as the percentage increase over the rate in the absence of any agonist: 1.01 ± 0.13 and 1.06 ± 0.14 (percent of total [^{3}H]phosphatidylcholine in the medium) in the pyrilamine and TCl experiments respectively. The effect of terbutaline alone was significantly different from that of the combination with pyrilamine ($P < 0.005$) and TCl ($P < 0.05$) when the data were analyzed with Student's two-tailed *t*-test for paired samples.

To determine if the observed stimulatory effects on secretion were due to reversal of an inhibitory effect of histamine, we examined the effect of histamine itself on both basal and terbutaline-stimulated phosphatidylcholine secretion. Histamine had no effect on either basal secretion (Table 1) or terbutaline-stimulated secretion (Fig. 4) in the range 10^{-9}–10^{-5} M. At the concentration of terbutaline used, its stimulatory effect was antagonized by the β-antagonist propranolol (Fig. 4).

DISCUSSION

There is one early report that the phenothiazide thiazinamium increases lung surfactant production. Krishnan *et al.* [16] found that thiazinamium methyl sulfate decreases the surface tension of lung extracts when administered intramuscularly to adult rats. The

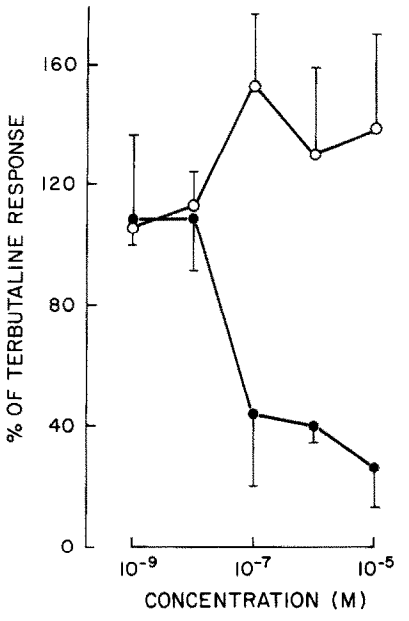

Fig. 4. Effects of histamine (○) and propranolol (●) on terbutaline-stimulated phosphatidylcholine secretion in type II cells. The cells were incubated with 10^{-5} M terbutaline and the indicated concentration of histamine or propranol for 90 min after which phosphatidylcholine secretion was measured. The data are expressed as a percentage of the stimulation produced by terbutaline alone and are means ± SE (bar) from four experiments. Control values are given in the legend of Fig. 1.

present report, however, is the first that thiazinamium chloride stimulates phosphatidylcholine secretion in cultures of isolated type II pneumocytes.

TCl stimulated secretion maximally to about half the extent of the β-agonists. The response of the type II cells to β-adrenergic agonists in the present study was similar to that reported by previous workers [6, 7]. Terbutaline and isoproterenol approximately doubled that rate of phosphatidylcholine secretion which is similar to the increase reported by Brown and Longmore [6] and Dobbs and Mason [7]. The EC_{50} values for terbutaline, 1.6×10^{-7} M, and isoproterenol, 3.2×10^{-9} M, also compare well to the values reported previously for terbutaline, 8×10^{-7} M [7], and isoproterenol, 1×10^{-9} M [6] and 4×10^{-9} M [7].

The EC_{50} for the stimulatory effect of TCl on phosphatidylcholine secretion in type II cells, 2.9×10^{-8} M, was similar to the EC_{50}, 5.1×10^{-8} M, reported for its antihistaminic action on guinea pig tracheal smooth muscle relaxation [1] and to the IC_{50}, 6.6×10^{-8} M, reported for its inhibitory effect on zymosan-induced thromboxane synthesis in rat alveolar macrophages [2]. The EC_{50} for the anticholinergic action of TCl in the tracheal smooth muscle system was 1.1×10^{-6} M [1]. The potency of TCl in stimulating phosphatidylcholine secretion in type II cells is therefore similar to its potency as an antihistaminic agent and as an inhibitor of thromboxane synthesis but greater than that of its anticholinergic action. An anticholinergic mechanism for this action of TCl would be surprising since cholinergic agonists are known to stimulate surfactant secretion *in vivo* [3, 4]. This effect of cholinergic agents on the lung, however, is thought to be mediated indirectly via the adrenal medulla since they do not stimulate phosphatidylcholine secretion in cultured type II pneumocytes [6, 7] or in adrenalectomized animals [17], and their effect in intact animals is blocked by the β-antagonist propranolol [17, 18]. That the stimulatory effect of TCl on phosphatidylcholine secretion is due to its inhibitory action on thromboxane synthesis would also be surprising since thromboxane has been reported to increase alveolar surfactant *in vivo* [19]. Other products of arachidonic acid metabolism have also been reported to stimulate phosphatidylcholine secretion [3, 4, 9, 10].

The fact that the structurally similar antihistamine promethazine as well as the structurally dissimilar antihistamine pyrilamine also stimulated phosphatidylcholine secretion suggests that the effect of TCl in type II cells is related to its antihistaminic property. Since the effects of TCl and pyrilamine were additive to the effect of terbutaline, it is likely that the antihistamines and β-agonists act via different mechanisms.

To explain the effect of TCl in terms of its antihistaminic property would require the surfactant secretion to be normally inhibited by histamine. In our system, histamine itself had no effect on phosphatidylcholine secretion. It is possible, however, that secretion was maximally inhibited by histamine present in the culture medium. Although it is unlikely that the type II cells were contaminated with mast cells, the culture medium contained L-histidine which is the immediate biosynthetic precursor of histamine. Further studies are needed to establish a role for histamine in regulation of surfactant secretion.

Acknowledgements—This work was supported by grants from Wyeth Laboratories and the National Heart, Lung and Blood Institute (HL-31175).

REFERENCES

1. A. J. Lewis, A. Dervinis and M. E. Rosenthale, *Eur. J. Pharmac.* **80**, 171 (1982).
2. J. Chang, S. Piperata, M. Skowronek and A. J. Lewis, *Biochem. Pharmac.* **32**, 2671 (1983).
3. S. A. Rooney, *Am. Rev. resp. Dis.* **131**, 439 (1985).
4. M. Hollingsworth and A. M. Gilfillan, *Pharmac. Rev.* **36**, 69 (1984).
5. A. M. Gilfillan, A. J. Chu, D. A. Smart and S. A. Rooney, *J. Lipid Res.* **24**, 1651 (1983).
6. L. A. S. Brown and W. J. Longmore, *J. biol. Chem.* **256**, 66 (1981).
7. L. G. Dobbs and R. J. Mason, *J. clin. Invest.* **63**, 378 (1979).
8. W. R. Rice, W. M. Hull, C. A. Dion, B. A. Hollinger and J. A. Whitsett, *Expl. Lung Res.* **9**, 135 (1985).
9. A. M. Gilfillan and S. A. Rooney, *Biochim. biophys. Acta* **833**, 336 (1985).
10. A. M. Gilfillan and S. A. Rooney, *Biochim. biophys. Acta* **876**, 22 (1986).
11. S. A. Rooney and A. M. Gilfillan, *Fedn. Proc.* **45**, 1665 (1986).
12. K. Sano, D. R. Voelker and R. J. Mason, *J. biol. Chem.* **260**, 12725 (1985).
13. A. M. Gilfillan, A. J. Lewis and S. A. Rooney, *Fedn. Proc.* **45**, 1665 (1986).

14. A. M. Gilfillan, A. J. Chu and S. A. Rooney, *Biochim. biophys. Acta* **794**, 269 (1984).
15. S. A Rooney, in *Handbook of Experimental Pharmacology* (Eds. H. P.Witschi and J. D. Brain), Vol. 75, p. 471. Springer, Berlin (1985).
16. B. Krishnan, K. Veeraraghavan and T. K. Nambinarayanan, *Curr. Sci.* **41**, 252 (1972).
17. M. M. Abdellatif and M. Hollingsworth, *Pediat. Res.* **14**, 916 (1980).
18. A. J. S. Corbet, P. Flax and A. J. Rudolph, *J. appl. Physiol* **43**, 1039 (1977).
19. M. J. Oyarzun, P. Donoso, M. Arias and D. Quijada, *Respiration* **46**, 231 (1984).