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Effects of the selective muscarinic receptor antagonist penehyclidine hydrochloride on the respiratory tract

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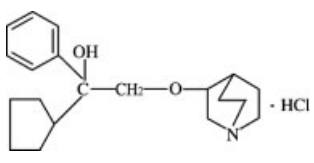
We investigated the effects and mechanisms of penehyclidine hydrochloride (PHC), a novel selective anticholinergic drug on respiratory tract. The methods of isolated guinea-pig tracheas, and isolated bronchoalveoar lavage fluid were employed to estimate PHC's anti-spasm mechanisms in smooth muscle. The results indicated relaxing effect of penehyclidine hydrochloride was obviously stronger than that of atropine sulfate in two assays according to PA_2 values and maximal reducing mount index. The method of radio-immunity assay was furtherly employed to determine cAMP levels in isolated guinea-pig tracheal and lung smooth muscle for comparing with selective effect on muscarinic receptor subtypes. In the assay, PHC could increase the content of cAMP in isolated guinea-pig lung smooth muscle, while decrease the content of cAMP in isolated guinea-pig tracheal smooth muscle, but the difference was no statistical significant at dose of 10 $\mu mol \cdot L^{-1}$. In conclusion, our results suggested that PHC has little or no effect on M_2 receptor subtypes in isolated guinea-pig tracheal and lung smooth muscle and could be used in asthma and COPD therapy.

1. Introduction

Penehyclidine hydrochloride (PHC, 8018), (2-hydroxyl-2-cyclopentyl-2-phenyl-ethoxy)quinuclidine, is an anticholinergic drug, which has both anti-muscarinic and anti-nicotinic activities and retains potent central and peripheral anti-cholinergic activities (Han et al. 2005). PHC has wide clinical use at present in China as an antagonist of organophosphate and soman poisoning (Wang et al. 2005; Li et al. 2003). However, anticholinergic agents also have other important uses as bronchodilators for the treatment of obstructive airway diseases, both asthma and, more particularly, chronic obstructive pulmonary disease (COPD) (Gross, 2006). In previous studies, we have investigated the anti-asthmatic effects of penehyclidine hydrochloride on guinea pigs and rabbits in vivo, the results indicating that the anti-asthmatic effect of penehyclidine hydrochloride aerosol ($0.50 mg \cdot ml^{-1}$) was equivalent to that of isoprenaline ($0.50 mg \cdot ml^{-1}$) (Xiao et al. 2006). So in this study, we further investigated the effects and mechanisms of penehyclidine hydrochloride on the respiratory tract *in vitro*.



Penchyclidine hydrochloride

At present, muscarinic receptors have been classified into five major subtypes: M_1, M_3 and M_5 (associated with the stimulation of phosphoinositide hydrolysis), and M_2 and M_4 (connected with the inhibition of adenylate cyclase) based on pharmacological and signal transductional criteria. The functional properties and binding profiles of the muscarinic M_1, M_2, M_3, M_4 and M_5 receptors closely correspond to those of the m_1, m_2, m_3, m_4 and m_5 receptor subtypes, which have been identified in recent receptor cloning studies. In guinea-pig tracheal and lung smooth muscle, the expression of at least two muscarinic receptor subtypes, M_2 and M_3, has been established (Roffel et al. 2001). Stimulation of muscarinic M_2 receptors decreases adenylyl cyclase activity via a Gi protein, thereby lowering cAMP levels (Sankary et al. 1988; Yang et al. 1991; Schaefer et al. 1995). On the other hand, stimulation of muscarinic M_3 receptors causes phosphoinositide hydrolysis via a Gq protein, thereby increasing production of inositol 1,4,5-trisphosphate and diacylglycerol (Roffel et al. 1990). Thus, muscarinic M_2 receptors contribute to negative regulation of intracellular cAMP levels and muscarinic M_3 receptors play an important role in the development of airway contraction. In addition, loss of M_2 receptor function increases acetylcholine release and potentiates vagally mediated bronchoconstriction, and these receptors are also dysfunctional in some patients with asthma (Minette et al. 1989). Though the receptor binding assay showed that PHC had far greater selectivity for M_3 over the M_1 receptor subtype (Niu et al. 1990), little is known about the effect of PHC on M_2 receptors.

In the present series of experiments, we clarified the effects of PHC on the M_2 receptors of the guinea-pig respiratory tract, employing isolated guinea-pig tracheas and isolated bronchoalveoar lavage fluid, while radio-immuno assay methods were also used.

2. Investigations and results

2.1. Isolated guinea-pig trachea assay

Table 1 shows the anti-bethanechol effect of PHC on isolated guinea-pig tracheas according to PA_2 values. Compared with the atropine sulfate group, PA_2 values with penehyclidine hydrochloride and gallamine were larger ($P < 0.001$), which indicates that the relaxant effects of penehyclidine hydrochloride and gallamine were clearly stronger than that of atropine sulfate in the isolated guinea-pig trachea anti-bethanechol assay.

Figures 1–3 show the concentration-response curves of bethanechol chloride in the absence or presence of atropine, PHC or gallamine in isolated guinea-pig trachea, respectively. Atropine, PHC and gallamine inhibited the contractile response of guinea-pig tracheal strips to bethanechol chloride in a concentration-dependent manner. The three drugs displaced the concentration-response curves to bethanechol chloride to the right and partly parallel.

Table 1: Effect of PHC on isolated guinea-pig trachea ($\bar{X} \pm S$ $n = 6$)

Drug	Samples	PA_2 anti-bethanechol values	P values
Atropine	6	8.9342 ± 0.13	
Gallamine	6	$10.3972 \pm 0.08^{**}$	$P < 0.001$
PHC	6	$11.4245 \pm 0.21^{**}$	$P < 0.001$

Compared with atropine group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

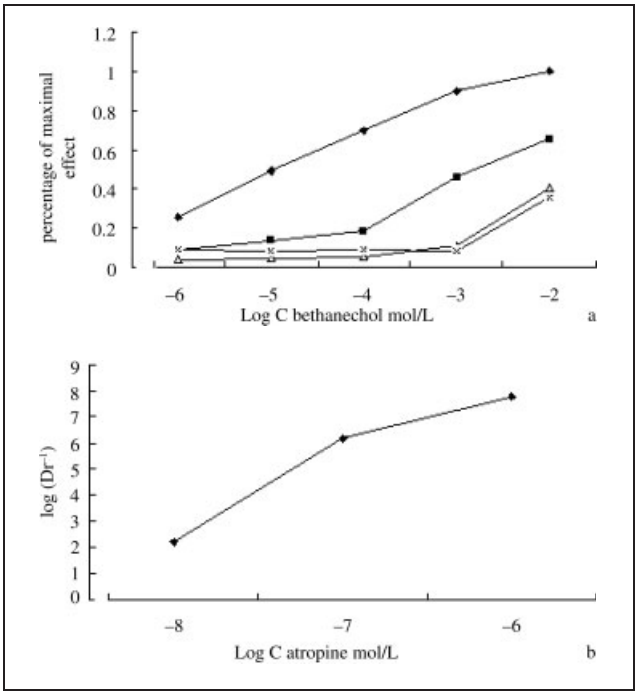


Fig. 1: Concentration-response curves of bethanechol chloride in the absence or presence of atropine sulfate in isolated guinea-pig trachea (a). Schild regression of atropine sulfate on isolated guinea-pig trachea (b). Data points represent the mean of four to six experiments. Standard errors were <10%. —◆— bethanechol chloride; —■— 10^{-8} mol/L atropine sulfate; —△— 10^{-7} mol/L atropine sulfate; —×— 10^{-6} mol/L atropine sulfate

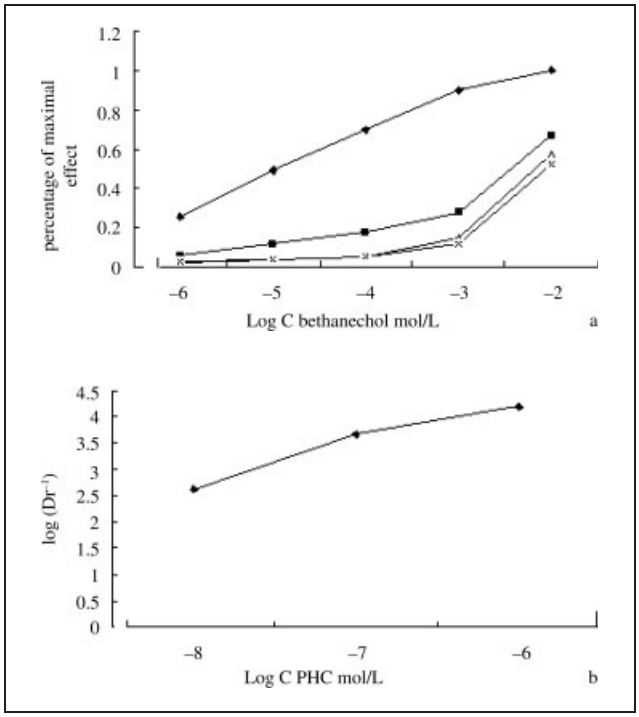


Fig. 2: Concentration-response curves of bethanechol chloride in the absence or presence of penehyclidine hydrochloride in isolated guinea-pig trachea (a). Schild regression of penehyclidine hydrochloride on isolated guinea-pig trachea (b). Data points represent the mean of four to six experiments. Standard errors were <10%. —◆— bethanechol chloride; —■— 10^{-8} mol/L penehyclidine hydrochloride; —△— 10^{-7} mol/L penehyclidine hydrochloride; —×— 10^{-6} mol/L penehyclidine hydrochloride

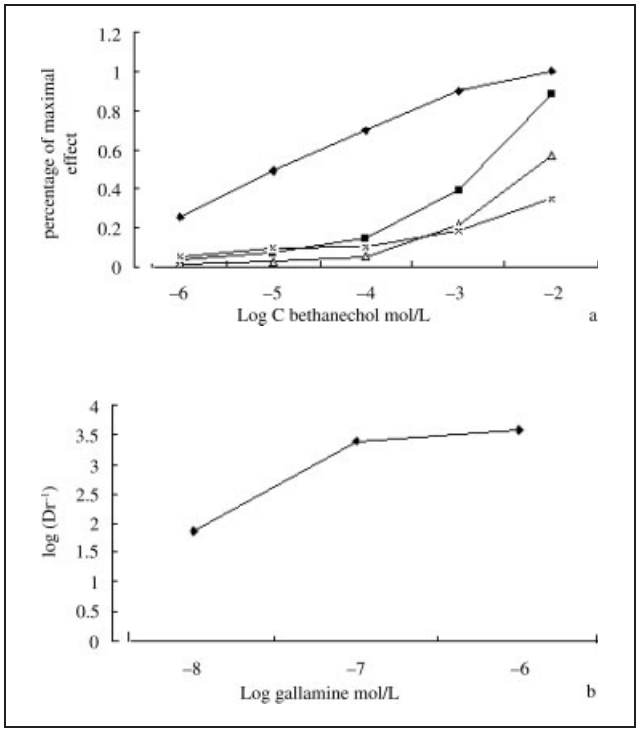


Fig. 3: Concentration-response curves of bethanechol chloride in the absence or presence of gallamine in isolated guinea-pig trachea (a). Schild regression of gallamine on isolated guinea-pig trachea (b). Data points represent the mean of four to six experiments. Standard errors were <10%. —◆— bethanechol chloride; —■— 10^{-8} mol/L gllamine; —△— 10^{-7} mol/L gallamine; –×–10^{-6} mol/ L gallamine

Table 2: Effects of PHC on guinea-pig isolated bronchoalveoar lavage fluid amount ($\bar{X} \pm S$ n = 6)

Groups	Drug (g/100ml)	average flow ml/min		maximal reduce amount	stable flow time (min)
		Pre-drug	Post-drug		
1	0.01% bethanechol	30.2 ± 0.12	5.25 ± 0.21	25.1 ± 0.10	13
2	0.01% PHC + 0.01% bethanechol	30.1 ± 0.20	23.1 ± 0.30	7.0 ± 0.31***□	10
3	0.1% PHC + 0.01% bethanechol	29.7 ± 0.50	25.8 ± 0.40	3.9 ± 0.45***□□	8
4	1% PHC + 0.01% bethanechol	31.1 ± 0.34	28.2 ± 0.60	2.9 ± 0.30***□□□	5
5	0.01% Atropine + 0.01% bethanechol	29.8 ± 0.40	22.3 ± 0.64	7.5 ± 0.12***	9
6	0.01% Gallamine + 0.01% bethanechol	30.5 ± 0.23	24.6 ± 0.52	5.9 ± 0.22***	3

Compared with bethanechol group: *P < 0.05, **P < 0.01, ***P < 0.001
Compared with atropine group: □P < 0.05, □□P < 0.01, □□□P < 0.001

2.2. Isolated bronchoalveoar lavage fluid assay

Table 2 shows the effects of PHC on isolated bronchoalveoar lavage fluid amounts in guinea-pigs. The effect of PHC in decreasing the amount of bronchoalveoar lavage fluid was dose-dependent. With an increasing dose of PHC, the anti-bethanechol-induced bronchial contraction effect was also augmented, according to the maximal reducing mount index; compared with both the control group and the atropine sulfate group, the differences were statistically significant ($P < 0.001$), and shortening the time of recovery stable. The anti-bethanechol-induced bronchial contraction effect of gallamine also increased with the amount in the bronchoalveoar lavage fluid amount too, and the differences were also statistically significant ($P < 0.001$), and the recovery time was shorter.

2.3. Radio-immuno assay

Table 3 shows the effects of PHC on cAMP levels in isolated guinea-pig trachea and lung smooth muscle. At a dose of 10 $\mu mol \cdot L^{-1}$ PHC increased the content of cAMP in isolated guinea-pig lung smooth muscle, while decreasing the content of cAMP in isolated guinea-pig tracheal smooth muscle, but the difference was not statistically significant. However, at the same dose both acetylcholine and bethanechol decreased the content of cAMP in isolated guinea-pig tracheal and lung smooth muscle and the difference was statistically significant ($P < 0.05$); both atropine and gallamine increased the content of cAMP in isolated guinea-pig tracheal and lung smooth muscle and the difference was statistically significant ($P < 0.001$).

3. Discussion

The findings of this study demonstrate that penehyclidine hydrochloride has a strong dilating effect on the smooth muscle of the respiratory tract of guinea-pigs at a functional level from the two isolated assays. Under "physiological" conditions, the airway smooth muscle contraction induced by muscarinic receptor agonists including acetylcholine, bethanechol, carbachol and so on is mediated via muscarinic receptors, primarily the M_3 subtype. This notion is supported by a large number of pharmacological studies that focus on determining the potency of several subtype preferential muscarinic antagonists (Roffel et al. 2001; Racké and Matthiesen 2004). In agreement with the conclusion that muscarinic M_3 receptors are the predominant subtype mediating airway smooth muscle contraction, it was observed that broncho-constriction induced by vagal nerve stimulation was lost in mice deficient for muscarinic M_3 receptors. In contrast, mice deficient for muscarinic M_2 receptors showed even greater vagally-induced bronchoconstriction, most likely because of an enhanced acetylcholine release due to the lack of autoinhibitory muscarinic M_2 receptors (Fisher et al. 2004). However, *in vitro* experiments on airway tissue from mice deficient for muscarinic M_2, M_3 or both receptors indicated that both receptor subtypes are involved in airway contraction induced by muscarinic agonists, although the functional contribution of the muscarinic M_3 receptor was significantly larger (Stengel et al. 2000, 2002; Struckmann et al. 2003). Atropine is a non-selective antagonist of muscarinic receptors, while gallamine is a muscarinic M_2 receptor-selective antagonist (Michel et al. 1990), in the isolated guinea-pig trachea and isolated bronchoalveolar lavage fluid assays, and our results confirmed the above conclusion. In addition PHC has a different selective effect on muscarinic receptors from that of atropine and gallamine.

So the next use of radio-immuno assay is to clarify the effect of PHC on M_2 receptors, based on the previous experiments with PHC in China. Large efforts have been made to elucidate the signaling pathways involved in the "pharmaco-mechanical" coupling of muscarinic receptors. It is well established that muscarinic M_3 receptor-mediated activation of phospholipase C resulting in the formation of IP3 and DAG is a key event in the signaling cascade leading to airway smooth muscle contraction, while muscarinic M_2 receptors are linked to the inhibition of adenylate cyclase activity, and activation of these receptor subtypes lowers tissue cyclic AMP levels (Eglen et al. 1996). In our assays, both acetylcholine and bethanechol are agonists of muscarinic M_2 receptors, while both atropine and gallamine are antagonists of muscarinic M_2 receptors. At a dose of 10 $\mu mol \cdot L^{-1}$, both acetylcholine and bethanechol were able to significantly decrease the content of cAMP in isolated guinea-pig tracheal and lung smooth muscle, while both atropine and gallamine significantly increased the content of cAMP in isolated guinea-pig tracheal and lung smooth muscle also. These results also agreed with the above conclusion. Although PHC increased the content of cAMP in isolated guinea-pig lung smooth muscle, while decreasing the content of cAMP in isolated guinea-

Table 3: Effects of PHC on cAMP levels in isolated guinea-pig trachea and lung smooth muscle ($\bar{X} \pm S$ n = 6)

Drug (10 μmol/L)	cAMP pmol/g (tracheal)	cAMP pmol/g (lung)
Control	129.01 ± 4.63	161.64 ± 30.35
Ach	69.19 ± 32.87*	124.95 ± 45.50*
Bethanechol	84.30 ± 32.01*	41.09 ± 16.71***
Atropine	198.58 ± 11.19***	221.35 ± 41.47*
Gallamine	182.65 ± 14.76***	298.46 ± 50.12***
PHC	119.86 ± 26.74	179.17 ± 55.35

Compared with control group: *P < 0.05, **P < 0.01, ***P < 0.001

pig tracheal smooth muscle, the difference was not statistically significant. According to our results, we conclude that PHC has little or no effect on M_2 receptor subtypes in isolated guinea-pig tracheal and lung smooth muscle, and PHC has a more selective effect on muscarinic M_3 receptor subtypes than M_2 receptor subtypes.

On the other hand, the cholinergic nervous system plays an important role in asthma and COPD. Vagally mediated reflex bronchoconstriction is seen after virus infection, exposure to ozone, or inhalation of antigen. Dysfunction of inhibitory muscarinic M_2 receptors on the vagal nerve endings may cause an increase of acetylcholine release. Because of the increased reflex bronchoconstriction resulting from these triggers of asthma attacks, anticholinergics may be particularly useful in the treatment of acute asthma. Improved anticholinergic medications, including selective M_3 antagonists, may offer effective interruption of these reflexes (Jacoby et al. 2001). So PHC could be a selective M_3 antagonist, which makes it potentially useful in the treatment of respiratory disorders such as asthma and COPD. Moreover, on one hand, the effects and mechanism of PHC in a rat model of COPD and asthma have been investigated in our lab, and the results showed PHC to be a good drug for the treatment of COPD and asthma (unpublished data); on other hand, some clinical research results have demonstrated that PHC had good curative effects for pulmonary dysfunction of COPD (Tao et al. 2006).

In conclusion, this was a pioneer work on the effects and mechanisms of PHC on the respiratory tract. The results showed that PHC has a strong dilating effect on the smooth muscle of the respiratory tract and has little or no effect on M_2 receptor subtypes in isolated guinea-pig tracheal and lung smooth muscle. PHC may be a promising candidate for asthma and COPD treatment in the future.

4. Experimental

4.1. General

All protocols were approved by the institutional ethics committee. The guinea-pigs were bought from the Animal Center of Sichuan University, Chengdu, China. All reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo, USA) unless otherwise specified. Penehyclidine hydrochloride powder was donated by Chengdu List Pharmaceutical Co., Ltd, and was of 99.99% purity. All compounds were dissolved in normal saline (0.9% NaCl solution) before use.

4.2. Isolated guinea-pig trachea assay

Hartly guinea-pigs of either sex (weighing 300–400 g) from the Animal Center of Sichuan University, were used for all experiments. Contractile responses of tracheal rings were measured as previously described (Liao et al. 1997). In brief, after mounting in the organ-bath, the rings were allowed to equilibrate at 37 °C aerated with a mixture of 95% O_2 and 5% CO_2 for 60 min at optimal resting tension (1 g) while being rinsed with KH solution every 15 min. Isometric contractions were recorded by a biological and functional experimental system (BL-410) via a force-displacement transducer. After establishing a steady basal tone, cumulative concentration-response curves to bethanechol were constructed, using incremental doses in concentrations from 10^{-6} to 10^{-2} mol · L^{-1} at 1 log intervals. Concentration were increased once a sustained response to the previous concentration was reached. An interval of 45 min was then allowed during which the tissues were washed with KH solution.

The tracheal rings were incubated with antagonist (PHC, atropine and gallamine) for the last 5 min and a second concentration-response curve to bethanechol was constructed in the presence of the three antagonists respectively. Three concentrations (10^{-8}, 10^{-7}, 10^{-6} mol · L^{-1}) of the three antagonists were added, respectively. One or two concentrations of one antagonist only was used for each ring.

Antagonist activity (PA_2) against bethanechol-induced contraction was determined for each muscarinic receptor antagonist (PHC, atropine and gallamine) according to the procedure of Arunlakshana and Schild (1959).

4.3. Isolated bronchoalveoar lavage fluid assay

The method was in accordance with that reported previously (Benoy et al. 1975) with minor modifications. Briefly, the trachea and lungs were dissected free from the killed guinea-pig, and the trachea tied quickly to a plastic cannula which was connected to a three-way tap through which drug solutions were injected. Then Locke solution incubated in a 37 °C water bath gassed with a mixture of 95% O_2 and 5% CO_2 was passed down the trachea through the bronchi, escaped from the alveoli through scratches on the surface of the lungs and was collected in a funnel. It then passed through a silicon rubber tube and entered a float recorder. When the stable rate of flow (30 ml/min) was reached, the drugs could be given. 0.01% bethanechol was injected first, and the response including flow rate, reduction in amount and time of stable flow recorded for 15 min. The tissues were then washed with Locke solution to reach the stable flow rate (30 ml/min) again. This was followed by injection of the three drugs (PHC, atropine and gallamine), and the response to the drugs was recorded for 15 min. Doses of bethanechol and the three drugs were alternated in this way until the tissue failed to respond to the drugs.

4.4. Radio-immuno assay

To measure cAMP accumulation, all experiments were conducted in the presence of a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (300 μM). The protocol was in accordance with that reported previously (Birdsall et al. 1999) with minor modifications. Briefly, isolated fresh guinea-pig trachea and lung strips weighing approximately 40–50 mg were incubated in Krebs solution at 37 °C gassed with a mixture of 95% O_2 and 5% CO_2 for 30 min and then exposed to 10 μmol · L^{-1} acetylcholine, bethanechol, PHC, gallamine or atropine for 10 min, respectively. Strips just before the application of drugs were used as untreated controls. After incubation for the given times, the muscle strips were frozen quickly in liquid nitrogen and homogenized in alcohol solution with a Polytron. After centrifugation at 3500 rpm for 15 min, twice, the alcohol in the supernatant was removed and the cAMP in the supernatant was measured with a [^{125}I] cAMP Kit (Shanghai University of Traditional Chinese Medicine). Sample results were divided by the weight of the wet tissue, and the results were expressed in pmol/g for the tracheal and lung strips.

4.5. Statistical analysis

Values are expressed as mean ± SD. One-way ANOVA was employed to determine the difference among groups. Significance level was set at $P < 0.05$.

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