Contractile and phosphatidylinositol responses of rat trachea to anticholinesterase drugs

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Purpose: Some anticholinesterases (anti-ChE) such as neostigmine and pyridostigmine but not edrophonium, stimulate phosphatidylinositol (PI) response. Although a direct relationship was suggested between the increase in PI response and airway smooth muscle contraction, there are no data regarding the effects of anti-ChE drugs on airway smooth muscle. Thus, we examined the contractile properties and PI responses produced by anti-ChE drugs.

Methods: *Contractile response.* Rat tracheal ring was suspended between two stainless hooks in Krebs-Henseleit (K-H) solution. (1) Carbachol (CCh), anti-ChE drugs (neostigmine, pyridostigmine, edrophonium) or DMPP (a selective ganglionic nicotinic agonist) were added to induce active contraction. (2) The effects of 4-diphenylacetoxy-N-methyl-piperidine methobromide (4-DAMP), an M_3 muscarinic receptor antagonist, on neostigmine- or pyridostigmine-induced contraction of rat tracheal ring were examined. (3) Tetrodotoxin (TTX) was tested on the anti-ChE drugs-induced responses. *PI response.* The tracheal slices were incubated in K-H solution containing LiCl and ^3H myo-inositol in the presence of neostigmine or pyridostigmine with or without 4-DAMP, an M_3 muscarinic receptor antagonist. ^3H inositol monophosphate (IP_1) formed was counted with a liquid scintillation counter.

Results: Carbachol ($0.1 \mu\text{M}$), neostigmine ($1 \mu\text{M}$), pyridostigmine ($10 \mu\text{M}$) but not edrophonium or DMPP caused tracheal ring contraction. 4-DAMP, but not tetrodotoxin, inhibited neostigmine and pyridostigmine-induced contraction. Neostigmine- or pyridostigmine-induced IP_1 accumulation was inhibited by 4-DAMP.

Conclusions: The data suggest that anti-ChE drugs activate the M_3 receptors at the tracheal effector site.

Objectif : Certains anticholinestérasiques (anti-ChE) comme la néostigmine et la pyridostigmine, mais non l'edrophonium, stimulent la réponse du phosphatidylinositol (PI). Bien qu'on ait évoqué une relation directe entre l'augmentation de la réponse PI et la contraction des muscles lisses du conduit aérien, il n'y a pas de données concernant les effets de médicaments anti-ChE sur les muscles lisses des voies aériennes. Donc, nous avons étudié les propriétés contractiles et les réponses du PI produites par les médicaments anti-ChE.

Méthode : *Réponse contractile.* Des anneaux de trachée de rat ont été suspendus entre des crochets inox dans une solution de Krebs-Henseleit (K-H). (1) Du carbachol (CCh), des médicaments anti-ChE (néostigmine, pyridostigmine, edrophonium) ou DMPP (un agoniste nicotinique ganglionnaire sélectif) ont été ajoutés pour induire une contraction active. (2) Les effets du méthobromide 4-diphénylacétoxy-N-méthyl-pipéridine (4-DAMP), un antagoniste des récepteurs muscariniques M_3 , sur la contraction d'anneaux de trachée de rat induite par la néostigmine ou la pyridostigmine, ont été examinés. (3) La tétrodotoxine (TTX) a été évaluée lors des réponses induites par les médicaments anti-ChE. *Réponse PI.* Les tranches de trachée ont été mises en incubation dans la solution K-H contenant du LiCl et ^3H myo-inositol en présence de néostigmine ou de pyridostigmine, avec ou sans 4-DAMP, un antagoniste des récepteurs muscariniques M_3 . Le monophosphate ^3H inositol (IP_1) ainsi formé a été mesuré à l'aide d'un compteur à scintillation liquide.

Résultats : Le carbachol ($0.1 \mu\text{M}$), la néostigmine ($1 \mu\text{M}$), la pyridostigmine ($10 \mu\text{M}$) mais non l'edrophonium ou le DMPP, ont causé des contractions des anneaux de trachée. La 4-DAMP, contrairement à la tétrodotoxine, a inhibé les contractions induites par la néostigmine et la pyridostigmine. L'accumulation d' IP_1 induite par la néostigmine ou la pyridostigmine a été inhibée par la 4-DAMP.

Conclusion : Les données indiquent que les médicaments anti-ChE activent les récepteurs M_3 au site effecteur de la trachée.

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WHEN neostigmine is used to reverse neuromuscular blockade, it inhibits acetylcholine (ACh) metabolism at the muscarinic receptors of airway smooth muscle as well as at the nicotinic receptors of the neuromuscular junction. Thus, neostigmine may induce an increase in ACh in muscarinic receptors which results in bronchoconstriction. However, the mechanisms involved in anticholinesterase (anti-ChE) drug-induced bronchoconstriction have not been clarified.

In a previous study we demonstrated that accumulation of inositol monophosphate (IP_1), a degradation product of the phosphatidylinositol (PI) response, was stimulated by neostigmine and by pyridostigmine, not by edrophonium in rat tracheal slices, and that these increases were inhibited by atropine.¹ The results suggest that neostigmine and pyridostigmine stimulate PI response in the airway smooth muscle, while edrophonium does not. Although a direct relationship was suggested between the increase in PI response and airway smooth muscle contraction,² there are no data regarding the effects of anti-ChE drugs on airway smooth muscle. Thus, we examined the contractile properties and PI responses produced by anti-ChE drugs.

Materials and Methods

The studies were conducted under guidelines approved by the Animal Care Committee of Nagasaki University School of Medicine. Forty-six male Wistar rats (Charles River, Yokohama Japan) weighing 250–350 g were used for the experiments. The rats were anaesthetized with 50 mg·kg⁻¹ pentobarbital *ip* and the trachea was rapidly isolated.

Contractile response

The trachea was cut into 3-mm-wide ring segments with a McIlwain tissue chopper (The Mickle Laboratory Engineering, Gomshall England). The tracheal ring was suspended between two stainless hooks and placed in a 5-mL water-jacketed organ chamber (Kishimotoika, Kyoto Japan) containing Krebs-Henseleit (K-H) solution (composition in mM: NaCl 118, KCl 4.7, CaCl₂ 1.3, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, glucose 11, Na₂-EDTA 0.05). The solution was continuously aerated with O₂95%/CO₂5%, with pH maintained at approximately 7.4 and temperature at 37°C. Isometric tensions were measured using isometric transducer (Kishimotoika, Kyoto Japan) and changes in isometric force were recorded using a MacLab system (Milford, MA). The resting tension was adjusted periodically to 1.5 g during the equilibration period. The ring was washed every 15 min and re-equilibrated to baseline tension for 60 min (Time 0).

(1) At time 0, carbachol (CCh), anti-ChE drugs (neostigmine, pyridostigmine, edrophonium) or DMPP (a selective ganglionic nicotinic agonist) were added stepwise-cumulatively to induce active contraction at 0.1 μ M to 100 μ M concentration. Carbachol is not broken down by cholinesterase,³ so that its action is prolonged and it was used in place of ACh. After completion of experiments with edrophonium or DMPP, 1 μ M CCh was added to test the integrity of the preparation when negative results were obtained with edrophonium or DMPP.

(2) The effects of 4-diphenylacetoxy-N-methylpiperidine methobromide (4-DAMP), an M₃ muscarinic receptor antagonist, on neostigmine- or pyridostigmine-induced contraction of rat tracheal ring were observed. At time 0, neostigmine or pyridostigmine (10 μ M each in final concentration) was added and, 30 min later, ring relaxation was induced by stepwise cumulative additions of 4-DAMP from 1 nM to 10 nM in final concentrations.

(3) Since tetrodotoxin (TTX) blocks axonal conduction by preventing the increase in permeability to Na⁺ associated with the rising phase of the action potential, a response to anti-ChE drugs in the presence of TTX rules out direct activation of cholinergic receptors on postganglionic cells. TTX was tested on the anti-ChE drugs-induced responses. At time 0, TTX (1 μ M in final concentration)⁴ or none was added and 15 min later neostigmine or pyridostigmine (10 μ M each in final concentration) was added. Different anti-ChE drugs, a muscarinic antagonist, a selective ganglionic nicotinic agonist or TTX was tested on the different preparation.

PI response

The technique of Brown *et al.*⁵ was used. Inositol 1,4,5-trisphosphate (IP_3) is rapidly degraded into IP_1 which is recycled back to phosphatidylinositol via free inositol. Lithium inhibits the conversion of IP_1 to inositol. Thus, in the presence of Li⁺, the accumulation rate of IP_1 reflects the extent of PI response. We measured [³H] IP_1 in tracheal slices incubated with [³H]*myo*-inositol (Amersham, Tokyo Japan). The trachea was cut longitudinally and chopped into 1-mm-wide pieces with a McIlwain tissue chopper. Three pieces of the tracheal slice were placed in small flat-bottomed tubes and preincubated for 15 min in K-H solution containing 5 mM LiCl. The solution was continuously aerated with O₂95%/CO₂5%. An aliquot of 0.5 μ Ci [³H]*myo*-inositol was then added to each tube (final concentration 0.1 μ M in 300 μ l incubation volume) and the tubes were flushed with O₂95%/CO₂5%, capped, set in a shaking bath at 37°C and incubated for 30 min (Time 0).

We determined the effects of 4-DAMP on neostigmine- or pyridostigmine-induced IP_1 accumulation in rat tracheal slices. At time 0, varying doses (0-100 nM) of 4-DAMP were added to the suspension of tracheal slices and the tubes were flushed with O_2 95%/CO₂ 5% and, 15 min later, neostigmine or pyridostigmine (10 μ M in final concentration) was added. After an additional 60 min the reaction was stopped with 940 μ L chloroform:methanol (1:2 v/v). Chloroform and water were then added (310 μ L each) and the phases were separated by centrifugation with 90 g for 5 min. The [³H] IP_1 was separated from [³H]*myo*-inositol in the water phase of 750 μ L by column chromatography using Dowex AG 1-X8 resin (Bio Rad, Richmond CA) in the formate form. The "n" refers to the number of experiments and one experiment includes the mean value of duplicate results. The [³H] IP_1 formed in the tracheal slices was counted with a liquid scintillation counter and presented by becquerels (Bqs). The counts in Bqs of two samples were averaged and the average Bqs of the blank values (no slices present) were subtracted to obtain the experimental data.

Data were expressed as mean $\pm$ SEM. The results of repeated measures and multiple groups were analyzed by a two-way analysis of variance. Multiple pairwise comparisons between groups were assessed by Scheffe's test. A P value < 0.05 was considered significant.

Results

Carbachol caused tracheal ring contraction at a dose of ≥ 0.1 μ M. Also, neostigmine caused contraction at a dose of ≥ 1 μ M, as did pyridostigmine at ≥ 10 μ M, whereas edrophonium or DMPP did not cause any contraction (Figure 1). The response of the preparation to CCh after completion of experiments with edrophonium or DMPP was normal. The ED_{50} of CCh, neostigmine or pyridostigmine was 0.18 ± 0.07 μ M, 0.42 ± 0.09 μ M, 2.8 ± 0.9 μ M, respectively. The dose of 4-DAMP to exert ID_{50} of 10 μ M neostigmine- or pyridostigmine-induced contraction was 4.0 ± 0.9 nM, 2.0 ± 0.2 nM, respectively (Figure 2). Tetrodotoxin did not inhibit 10 μ M neostigmine- or pyridostigmine-induced contraction (Figure 3).

Neostigmine- or pyridostigmine-induced IP_1 accumulation was inhibited by 4-DAMP at a dose of 10 nM or greater (Figure 4). There were no differences between the basal value and values at 10 nM of 4-DAMP. The ID_{50} of 4-DAMP on neostigmine- or pyridostigmine-induced IP_1 accumulation was 4.3 ± 0.4 nM and 1.3 ± 0.3 nM, respectively. These data are consistent with those on neostigmine- or pyridostigmine-induced contraction, respectively $r = 0.938$, $P < 0.01$ for neostigmine, and $r = 0.933$, $P < 0.01$ for pyridostigmine).

FIGURE 1 Effects of carbachol (CCh), anticholinesterase (anti-ChE) drugs or a selective ganglionic nicotinic agonist, 1,1-dimethyl-4-phenyl-piperazinium (DMPP) on resting tension of rat trachea (mean $\pm$ SEM; $n = 6-10$). * $P < 0.05$, † $P < 0.001$ vs 0.

FIGURE 2 Effects of 4-DAMP, M_3 muscarinic receptor antagonist on 10 μ M neostigmine- or 10 μ M pyridostigmine-induced tension of rat trachea (mean $\pm$ SEM; $n = 7$). * $P < 0.001$ vs 4-DAMP 0.

Discussion

The main findings from our present study are that carbamyl-containing anti-ChEs, neostigmine and pyridostigmine, produce dose-dependent increases in the tracheal contractile responses which are blocked by 4-DAMP. Edrophonium, which lacks a carbamyl group,

FIGURE 3 Effects of 1 μ M tetrodotoxin (TTX) on 10 μ M neostigmine- or 10 μ M pyridostigmine-induced tension of rat trachea (mean $\pm$ SEM; $n = 6$). TTX(+): TTX 1 μ M.

FIGURE 4 Effects of 4-DAMP, M_3 muscarinic receptor antagonist on 10 μ M neostigmine- or pyridostigmine- induced IP_1 accumulation of rat trachea (mean $\pm$ SEM; $n = 6-9$).

* $P < 0.01$, † $P < 0.001$ vs 4-DAMP 0.

has no effect on the contractile response. The data suggest that anti-ChE drugs have different effects on airway smooth muscle contraction. In our previous study, accumulation of IP_1 was stimulated by neostigmine and pyridostigmine in a dose-dependent manner but was not affected by edrophonium.¹ These PI responses are consistent with the contractile responses by anti-ChE drugs in the present study. Thus, the difference of contraction would be accounted by the effects on the PI response.

FIGURE 5 Schematic diagram of the peripheral tracheal parasympathetic pathway.

Although the mechanism involved in the differential effects of anti-ChE drugs on airway smooth muscle has not been clarified, the following explanations may be advanced (Figure 5):

1) Anti-ChE drugs may stimulate ACh receptors of postganglionic neurons, resulting in an increase in ACh release from postganglionic nerve terminals. Riker and Wescoe⁶ reported that neostigmine produced skeletal muscle contraction in the cat, even after complete inactivation of acetylcholinesterase. Sherby *et al.*⁷ observed that anti-ChE drugs were bound to nicotinic ACh receptors in rat skeletal muscle, and that neostigmine and pyridostigmine acted as partial agonists. Carlyle⁸ demonstrated that neostigmine produced an atropine-sensitive contraction of the guinea pig trachealis muscle, which was abolished by maneuvers inhibiting release of ACh from nerve terminals (i.e., local anaesthetics, cooling and hemicholinium). These data suggest activation by anti-ChE drugs of nicotinic receptors on postganglionic neurons, which are different from nicotinic receptors of neuromuscular junction. Backman and colleagues⁹⁻¹² reported that neostigmine could activate muscarinic receptors in the parasympathetic postganglionic neurons in the heart. Since parasympathetic postganglionic neurons are considered to be close to

the targeted end-organ, tracheal rings used in the present study would contain parasympathetic postganglionic neurons. However, DMPP did not cause contraction. The equipment was sensitive enough to measure contractions produced by anti-ChE drugs or CCh, so the inability to detect contractions caused by nicotinic agonists is not obviously related to equipment insensitivity. More likely, the preparation does not appear to contain a sufficient number of functional postganglionic cells which can be activated by the nicotinic agonists. Thus, we cannot conclude that anti-ChE drugs do not activate ACh receptors on postganglionic neurons.

2) ACh may accumulate at the endplate of airway smooth muscle, resulting from inhibition of cholinesterase activity. Randall *et al.*¹³ reported that pyridostigmine had 1/20, and edrophonium had 1/200 the potency of neostigmine to inhibit red blood cell cholinesterase. However, in the present study even maximal concentrations of edrophonium did not cause a contractile response compared with that by neostigmine or pyridostigmine. The lack of effect by edrophonium suggests that there is no spontaneous release. The lack of effect by edrophonium may be secondary to a lack of a functional postganglionic neurons as the DMPP experiment suggests. Thus, the differential effects of anti-ChE drugs could not be explained by this mechanism.

3) Anti-ChE drugs may act directly on muscarinic receptors of airway smooth muscle cell membrane. The neostigmine-induced bradycardia might, in part, be due to direct activation of muscarinic receptors within parasympathetic ganglion.⁹⁻¹² Thus, it is possible that some anti-ChE drugs would stimulate directly muscarinic receptors on airway smooth muscle cell membrane, resulting in inducing contractile response.

Muscarinic ACh receptors in the airway are divided into M_2 and M_3 receptors.^{14,15} The M_3 muscarinic receptors exist on airway smooth muscle cell membrane, and M_2 muscarinic receptors exist on postganglionic nerve terminals. Stimulation of M_3 receptors induces bronchoconstriction, whereas stimulation of M_2 receptors inhibits ACh release, resulting in attenuation of vagally-induced bronchoconstriction. In the present study, the contraction by neostigmine or pyridostigmine of rat tracheal rings was completely inhibited by 4-DAMP, a selective M_3 antagonist at a dose of 10 nM. Ten Berge *et al.*¹⁶ tested the effects of 4-DAMP on the twitch response of electrical field-stimulated guinea pig tracheal ring preparations, and found that twitch contraction was nearly inhibited by 4-DAMP at a dose of 10 nM. Their result is consistent with our data. Thus, in the present study the contrac-

tile response to anti-ChE drugs is likely to be mediated via M_3 muscarinic receptors.

Peak serum concentrations of neostigmine, pyridostigmine and edrophonium were 0.9 μ M, 5 μ M and 50 μ M during reversal of neuromuscular blockade by 0.07 mg·kg⁻¹ neostigmine, 0.35 mg·kg⁻¹ pyridostigmine and 10 mg·kg⁻¹ edrophonium, respectively.¹⁷ Thus, the concentrations used in the present study are within the clinical range. The muscarinic side-effects of neostigmine and pyridostigmine may be due, in part, to direct activation of muscarinic receptors in the peripheral parasympathetic nervous system.

In conclusion, the data suggest that the anti-ChE drugs activate the M_3 receptors at the effector site. However, we cannot eliminate the possibility that these drugs do not activate receptors on the postganglionic neurons.

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