Effect of 1,1-dimethylphenyl 1,4-piperazinium on mouse tracheal smooth muscle responsiveness

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Effect of 1,1-dimethylphenyl 1,4-piperazinium on mouse tracheal smooth muscle responsiveness. Am J Physiol Lung Cell Mol Physiol 288: L1139–L1145, 2005. First published February 4, 2005; doi:10.1152/ajplung.00406.2004.—Bronchial hyperresponsiveness is one of the main features of asthma. A nicotinic receptor agonist, 1,1-dimethylphenyl 1,4-piperazinium (DMPP), has been shown to have an inhibitory effect on airway response to methacholine in an in vivo model of asthma. The aims of this study were to 1) verify whether nicotinic acetylcholine receptors (nAChR) were present on mouse tracheal smooth muscle, 2) verify whether bronchoprotection observed in mice was due to a direct effect on airway smooth muscle, and 3) compare the effects of nicotinic agonists to that of salbutamol. α3-, α4-, and α7-nAChR subunits were detected by immunofluorescence on tracheal tissues from normal BALB/c mice. The effect of DMPP on tracheal responsiveness was verified by an isometric method. Tracheas were isolated from normal mice, placed in organ baths, and contracted with a single dose of methacholine. Cumulative doses of DMPP or salbutamol were added to the baths. Results show that mouse tracheal smooth muscle is positive for α3- and α7-nAChR subunits and that the epithelium is positive for α3-, α4-, and α7-subunits. DMPP induced a greater dose-dependent relaxation of tracheal smooth muscles precontracted with methacholine than with salbutamol. These results suggest that the smooth muscle-relaxing effect of DMPP could have some interest in the treatment of obstructive pulmonary diseases.

AIRWAY RESPONSIVENESS; SALBUTAMOL; NICOTINIC AGONISTS

NICOTINIC RECEPTORS ARE EXPRESSED ON NEURONAL AND DIFFERENT TYPES OF NONNEURONAL CELLS. THESE RECEPTORS MEDIATE NUMEROUS SPECIFIC PHYSIOLOGICAL FUNCTIONS DEPENDING ON THE TYPE OF CELL INVOLVED. FOR EXAMPLE, NICOTINE INDUCES THE RELEASE OF Dopamine IN THE CENTRAL NERVOUS SYSTEM AND STIMULATES NITRIDE OXIDE (NO) SYNTHASE GENE EXPRESSION IN ENDOTHELIAL CELLS (1, 15). IN THE AIRWAYS, NICOTINIC RECEPTORS ARE FOUND ON NERVE CELLS, EPITHELIAL CELLS, ALVEOLAR MACROPHAGES, AND T LYMPHOCYTES (5, 9, 11). NICOTINIC RECEPTORS HAVE BEEN DESCRIBED ON RAT ARTERIAL SMOOTH MUSCLE CELLS (3), BUT IT REMAINS UNKNONW WHETHER THEY ARE PRESENT ON AIRWAY SMOOTH MUSCLE CELLS.

THE EFFECT OF STIMULATING NICOTINIC ACETYLCHOLINE RECEPTORS (nAChRs) BY SPECIFIC NICOTINIC ACETYLCHOLINE RECEPTOR AGONISTS (nAChRAs) IN THE LUNG IS CONTROVERSIAL. ACTIVATION OF NICOTINIC RECEPTORS ON AIRWAY NEURONAL CELLS INDUCES THE RELEASE OF ACETYLCHOLINE BY CHOLINERGIC NERVES, CAUSING A BRONCHOCONSTRICTION THROUGH STIMULATION OF MUSCARINIC RECEPTORS (14). HOWEVER, THEIR STIMULATION CAN ALSO HAVE A BRONCHODILATORY EFFECT BY INDUCING THE RELEASE OF RELAXING FACTORS BY EITHER THE ADRENERGIC OR THE NONADRENERGIC NONCHOLINERGIC NERVOUS SYSTEM (14). ACTIVATION OF NICOTINIC RECEPTORS ON AIRWAY NONNEURONAL CELLS COULD ALSO CONTRIBUTE TO THE EFFECT OF nAChRA ON BRONCHOMOTOR TONE (8).

OUR GROUP (2) HAS RECENTLY SHOWN THAT 1,1-DIMETHYLPHENYL 1,4-PIPERAZIUM (DMPP) INDUCES A DECREASE IN AIRWAY RESISTANCE IN VIVO IN OVALBUMIN-SENSITIZED MICE. THIS OVERALL EFFECT COULD HAVE ORIGINATED FROM NERVE STIMULATION, FROM DIRECT SMOOTH MUSCLE STIMULATION, OR VIA THE RELEASE OF SMOOTH MUSCLE-RELAXING MEDIATORS BY EPITHELIAL OR INFLAMMATORY CELLS PRESENT IN THE LUNG. THE PRESENT STUDY WAS DONE IN AN ATTEMPT TO VERIFY WHETHER nAChRs ARE PRESENT ON AIRWAY SMOOTH MUSCLE AND WHETHER nAChRAs HAVE A DIRECT EFFECT ON AIRWAY SMOOTH MUSCLES, WHICH COULD EXPLAIN, AT LEAST IN PART, THE OBSERVED BRONCHODILATORY EFFECT IN VIVO. A THIRD OBJECTIVE WAS TO COMPARE THE SMOOTH MUSCLE-RELAXING EFFECT OF nAChRA TO THAT OF SALBUTAMOL, A β2-ADRENERGIC RECEPTOR AGONIST COMMONLY USED IN THE TREATMENT OF ASTHMA.

MATERIALS AND METHODS

Animals and preparation of tracheal rings. Female BALB/c mice (18–20 g, 6–8 wk old) were purchased from Charles River (St-Constant, PQ, Canada). The protocol was approved by our institution’s ethics committee and conducted according to the Helsinki Conventions. Mice were killed by an intraperitoneal injection of 0.1 ml of pentobarbital sodium (65 mg/ml Somnotol, MTC Pharmaceuticals, Cambridge, ON, Canada). Tracheas were then excised and either frozen in OCT (immunofluorescence studies), placed in 4% parafomaldehyde (histological studies), or placed in Krebs bicarbonate solution (isometric studies). Immunofluorescence studies. Immunofluorescence technique was performed on 6-μm-thick sections mounted on Surgipath slides. Slides were fixed in acetone-methanol solution (60/40) for 10 min at −20°C. OCT was removed by soaking the slides in Tris-buffered saline for 10 min at room temperature. Tissue sections were incubated with the Dako protein block serum-free solution for 30 min at 37°C. We detected α3-, α4-, and α7-nAChR subunits by an indirect staining method using specific rabbit polyclonal antibodies, which was followed by incubation with Alexa fluor 488-labeled anti-rabbit IgG (excitation 450–490 nm, emission 520 nm). Adjacent slides were stained with a Cy3-labeled antibody (excitation 510–560 nm, emission 590 ± 20 nm) directed against smooth muscle α-actin. Double staining was also performed on some slides; however, some interference was observed between the two stainings. Control slides were preincubated with Tris-buffered saline (negative control) or a rabbit IgG from Dako Cytomation (isotypic control) instead of nAChR subunit–specific antibodies. Incubations with all antibodies were performed at 37°C for 30 min. To diminish background staining, some slides were soaked in 0.2% Evans blue (Sigma-Aldrich, St. Louis, MO).

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Histological studies. Tracheal tissue was first fixed in a 4% paraformaldehyde solution and embedded in paraffin. Ten-micrometer-thick tissue sections were then sliced and mounted on slides. Hematoxylin-eosin staining was performed on slices and studied under light microscopy.

Antibodies. As stated in the technical sheets provided by the company, all antibodies used for detection of nAChR subunits were rabbit polyclonal antibodies raised against different recombinant proteins (RP) (see Table 1). Anti-α3 was raised against a RP corresponding to amino acids 367–502 mapping at the carboxy terminus of the α3-subunit of human origin. Anti-α4 was raised against a RP corresponding to amino acids 342–474 and anti-α7 against a RP corresponding to amino acids 367–502, all mapping at the carboxy terminus of their corresponding nAChR subunit of human origin.

Table 1. Antibodies used for detection of nicotinic acetylcholine receptor subunits

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Company</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Polyclonal anti-α3 nAChR subunit</td>
<td>Santa Cruz Biotechnology</td>
<td>Undiluted</td>
</tr>
<tr>
<td>Polyclonal anti-α4 nAChR subunit</td>
<td>Santa Cruz Biotechnology</td>
<td>Undiluted</td>
</tr>
<tr>
<td>Polyclonal anti-α7 nAChR subunit</td>
<td>Santa Cruz Biotechnology</td>
<td>Undiluted</td>
</tr>
<tr>
<td>Alexafluor 488-labeled goat anti-rabbit IgG</td>
<td>Molecular Probes</td>
<td>1:150</td>
</tr>
</tbody>
</table>
| Monoclonal anti-α-smooth muscle actin, clone 1A4, Cy3 Conjugate | Sigma | 1:25

nAChR, nicotinic acetylcholine receptor.
Data analyses. Results are expressed as means ± SE. We determined the statistical differences between mean values using an ANOVA unpaired t-test. A P value of <0.05 was considered significant.

RESULTS

Immunofluorescence. α3- (Fig. 1), α4- (Fig. 2), and α7-subunits (Fig. 3) were detected in the epithelial layer, whereas α4- and α7-subunits were also present in the smooth muscle.

Confirmations of locations of α3- (Fig. 1, A and B), α4- (Fig. 2, A and B), and α7-subunits (Fig. 3, A–C) were obtained by double staining with an α-actin-specific antibody. Single nAChR subunit stainings were also done on adjacent slides (Figs. 1D, 2D, and 3D). Negative and isotypic controls showed no reactivity (Fig. 4).

Isometric studies. Compared with controls, DMPP (10^{-4}–10^{-3} M) induced a dose-dependent relaxation of methacholine-
precontracted tracheal smooth muscles (Fig. 5; \( P < 0.0001, n = 15 \)). Only \( 10^{-4} \) and \( 10^{-3} \) M of DMPP induced significant relaxation.

The relaxing effect of DMPP was also compared with that of salbutamol. DMPP induced a significantly greater relaxation than salbutamol at high doses (\( 10^{-4}–10^{-3} \) M) (Fig. 6; \( P < 0.0001, n = 9 \)).

Hexamethonium (\( 10^{-1} \) M) completely inhibited the relaxation induced by DMPP (Fig. 7; \( P \leq 0.0001, n = 4 \)), whereas \( \alpha \)-bungarotoxin had no significant effect on DMPP-induced relaxation (Fig. 8; \( n = 6 \)).

Removing the epithelium partially inhibited the relaxation induced by DMPP (Fig. 9; \( P < 0.05, n = 6 \)). The effect of epithelial removal was significant at \( 10^{-4} \) M. When the same experiment was repeated with L-NAME, to inhibit NO production, relaxation was significantly inhibited (Fig. 10; \( P < 0.05 \) and \( P \leq 0.001, n = 6 \)) at all doses. The nerve blocker TTX did not significantly inhibit relaxation (Fig. 11; \( n = 5 \)).

**DISCUSSION**

This study demonstrates that DMPP is able to relax mouse tracheal rings. The effective DMPP concentrations were comparable to concentrations previously used in similar biological systems (8, 12). This relaxing effect can exceed that of salbutamol on mouse tracheal rings. Because salbutamol is a \( \beta_2 \)-adrenoceptor agonist and relaxation of murine tracheal smooth muscle is predominantly mediated by \( \beta_1 \)-adrenoceptors (4), these results must be regarded with caution. Additional experiments need to be done on other animal species and humans before we can confirm that the bronchodilation induced by DMPP is similar or even greater than that induced by salbutamol. The blocking effect of hexamethonium confirms that the relaxing effect is via nicotinic receptor activation. No significant inhibitory effect was obtained by the addition of \( \alpha \)-bungarotoxin. Therefore, \( \alpha_7 \)-nAChR subunit does not seem to be involved in the relaxing effect of DMPP. Immunofluorescence analyses showed the presence of \( \alpha_3 \), \( \alpha_4 \), and \( \alpha_7 \)-nAChR subunits in the epithelium, whereas \( \alpha_2 \)- and \( \alpha_7 \)-subunits were present in smooth muscle, suggesting that \( \alpha_3 \)- and \( \alpha_4 \)-receptor subunits could be involved in the relaxing effect of DMPP.

Similar smooth muscle relaxing effects of nAChRA have been previously obtained with isolated tracheas of pigs (8) and guinea pigs (13). Although never confirmed, several studies (13, 14) have suggested that relaxation could be induced by the stimulation of nicotinic receptors on smooth muscle cells. In our experiments, relaxation occurred even in the presence of the nerve blocker TTX. This supports the idea that DMPP...
could act directly through nAChRs localized on tracheal smooth muscle. Our results also show the presence of \( \alpha_4 \)- and \( \alpha_7 \)-nAChRs on tracheal smooth muscle cells, thus providing further evidence for a direct action on this cell type.

Studies by Kannan and Johnson (8) support the nonneuronal origin of the relaxation induced by nAChRA. They suggested that, instead of smooth muscle, other cell types such as epithelial cells could play a role in DMPP-induced relaxation. Our results show that epithelium is partially responsible for the DMPP effect. Removal of the epithelium modified the relaxation induced by 0.1 mM DMPP. NO release by epithelial cells could explain the relaxing effect of the epithelium. The effect obtained by pretreatment with L-NAME was similar to that obtained by epithelial removal, except for the highest dose, in which L-NAME showed a greater inhibitory effect than epithelial removal. Because NO is produced by both the epithelium and airway smooth muscle (10), the inhibitory effect of L-NAME could originate from both types of tissue. Also of note is that the lack of epithelium did not affect methacholine-induced precontraction; similar contractile tensions were obtained with the same doses of methacholine with epithelium-denuded preparations vs. those with intact epithelium (data not shown).

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Fig. 7. Dose-response curves for the relaxation of methacholine-precontracted BALB/c tracheas by DMPP with (●; \( n = 4 \)) or without (●; \( n = 14 \)) hexamethonium (Hexa; 0.1 M) pretreatment. Each point represents mean ± SE of the percentage of maximal contraction induced by DMPP. **** \( P \leq 0.0001 \), Hexa + DMPP compared with DMPP only.

Fig. 8. Dose-response curves for the relaxation of methacholine-precontracted BALB/c tracheas by DMPP with (●; \( n = 6 \)) or without (●; \( n = 14 \)) \( \alpha_7 \)-bungarotoxin (Bung) pretreatment and control tracheas (●; \( n = 5 \)). Each point represents mean ± SE of the percentage of maximal contraction induced by DMPP.

Fig. 9. Dose-response curves for the relaxation of methacholine-precontracted BALB/c tracheas by DMPP with (+) epithelium (●; \( n = 14 \)) and without (−) epithelium (●; \( n = 6 \)). Control curve. Each point represents mean ± SE of the percentage of maximal contraction induced by DMPP. * \( P < 0.05 \), tracheas with epithelium compared with tracheas denuded of epithelium.

Fig. 10. Dose-response curves for the relaxation of methacholine-precontracted BALB/c tracheas by DMPP with (●; \( n = 6 \)) or without (●; \( n = 14 \)) L-NAME pretreatment and control tracheas (●; \( n = 5 \)). Each point represents mean ± SE of the percentage of maximal contraction induced by DMPP. * \( P < 0.05 \) and *** \( P \leq 0.001 \), L-NAME + DMPP compared with DMPP only.
DMPP-induced in vivo bronchodilation in cats has been shown to be adrenergic in nature, whereas bronchodilations with nicotine and acetylcholine are a combination of adrenergic and nonadrenergic noncholinergic influences (14). These results are supported by other findings made in guinea pig isolated tracheas (7), in which nicotine-induced relaxation has been attributed to stimulation of adrenergic nerves. However, a direct effect of nicotinic stimuli on airway smooth muscle could not be excluded by that study. We did not test adrenergic antagonists in the present experiments.

Numerous studies have described a nAChRA-induced bronchoconstriction. Nicotine contracted bronchial preparations of guinea pig, rabbit, and crab-eating monkey ex vivo, whereas a biphasic response to nicotine was obtained in guinea pig tracheal preparations; this biphasic response consisted of an initial contraction followed by a large relaxation (7, 13). These contractile effects seem to be due to a nAChRA-induced release of acetylcholine, which then acts on muscarinic receptors to initiate contraction. In our study, the precontractions were obtained with methacholine, a muscarinic agonist. This could have prevented acetylcholine to act on muscarinic receptors and modify the smooth muscle response to nAChRAs. Experiments done on tracheal strips at basal tension however showed no contractile activity of DMPP.

Several other factors could explain opposite results of nAChRA on smooth muscle tone. Responses to nAChRA seem to vary greatly between different tissues. For example, Takeno et al. (13) found that nicotine contracted guinea pig bronchial preparations but relaxed tracheal preparations of the same animal. Given the small size of mice, bronchial strips could not be prepared in our set of experiments.

In addition, DMPP seems to have specific effects in different species. It has a relaxing effect on carbachol-precontracted isolated tracheal strips of pigs (8) but contracted mongrel dog tracheas (6). Our results demonstrate that DMPP is able to relax mouse tracheal tissue.

The mechanisms underlying the relaxation induced by nAChRA are still unclear. Our study suggests that relaxation could be mediated via a direct effect on the α4-nicotinic receptor subunit present on smooth muscle tissue or indirectly via NO production induced by specific activation of α3- or α4-subunits present on the epithelium or of α7-subunit present on the smooth muscle cells. On the basis of our results, the α7-subunit has a very limited effect. Further studies will be needed to identify other possible subunits implicated in the dilatory effects of DMPP. The identification of the subunits involved could lead to the development of more specific agonists for a more effective treatment of obstructive pulmonary diseases.

In conclusion, α3-, α4-, and α7-nicotinic receptor subunits are expressed on epithelial cells of mouse tracheas, whereas α4- and α7-subunits are expressed on tracheal smooth muscle cells. The relaxing effect of DMPP seems to be mediated by epithelial and smooth muscle cells. DMPP is therefore a nicotinic agonist that could have valuable bronchoprotective effects, and this molecule or other nAChRAs could be of interest in the treatment of asthma and other obstructive pulmonary diseases.

GRANTS
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DISCLOSURES
The data presented in this study are related to the pending patent PCT/CA02/00412 owned by Laval University. Y. Cormier and E. Israel-Assayag are two of the coinventors of that patent. Laval University has granted a licensing to Asmacure Ltd., a research and development company partly owned by Cormier and Israel-Assayag.

REFERENCES


