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

G. Dorion, E. Israë!-Assayag, M. J. Beaulieu, and Y. Cormier

Unité de Recherche en Pneumologie, Centre de Recherche de l’Hôpital Laval, Institut Universitaire de Cardiologie et de Pneumologie de l’Université Laval, Sainte-Foy, Québec, Canada

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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 α2-, α3-, 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.

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 NITRIC OXIDE SYNTHASE GENES IN ENDOTHELIAL CELLS. IN THE AIRWAYS, NICOTINIC RECEPTORS ARE FOUND ON NERVE CELLS, EPITHELIAL CELLS, ALVEOLAR MACROPHAGES, AND T LYMPHOCYTES. NICOTINIC RECEPTORS HAVE BEEN DESCRIBED ON RAT ARTERIAL SMOOTH MUSCLE CELLS, BUT IT REMAINS UNKNOWN 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. 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. Activation of nicotinic receptors on airway nonneuronal cells could also contribute to the effect of nAChRAs on bronchomotor tone.

Our group has recently shown that 1,1-dimethylphenyl 1,4-piperazinium (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 nAChRAs 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 or placed in 4% paraformaldehyde (histological studies), placed in Krebs bicarbonate solution (isometric studies). Female BALB/c mice (9, 11). Nicotinic receptors have been described on rat arterial smooth muscle cells, but it remains unknown whether they are present on airway smooth muscle cells.

Three objectives were set for this study: 1) verify whether nAChRs are 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 α2-, α3-, 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.

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thick tissue sections were then sliced and mounted on slides. Hematox- 
ilic formaldehyde solution and embedded in paraffin. Ten-micrometer-
sectioned tissue was then placed in Krebs solution (in mM: 118 NaCl, 4.7 KCl, 1.8
CaCl2, 25 NaHCO3, 2.5 CaCl2•H2O, 25 NaHCO3, and 5.6
D-glucose). Extreme care was taken not to stretch the rings, to keep the
smooth muscle tissue intact. Tracheal rings were mounted isometrically
to force transducers and equilibrated for 1 h in 5-ml organ baths
(Radnoti Four-Unit tissue bath system, Harvard Apparatus) containing
Krebs bicarbonate solution at pH 7.4, maintained at 37°C, and
a single dose of 120 mM KCl, which causes viable tissue to contract.
Preparations were then washed and allowed to reequilibrate for 15
min. After 15 min, the contraction obtained with KCl had waned and the
tension had returned to baseline values.

**Isometric experiments.** Tissues were first precontracted with a
submaximal (10−5 M) concentration of methacholine (American
Pharmaceutical Partners, Los Angeles, CA). The mean contraction
tension induced was 1,292 ± 433 mg/cm. When a plateau level of
contraction was reached, cumulative doses (from 10−8 to 10−3 M) of
DMPP (Sigma-Aldrich) or salbutamol (GlaxoSmithKline, Montreal,
PQ, Canada) were added to the baths, and the tensions were recorded.
The results are expressed as a percentage of the DMPP- or salbuta-
mol-induced relaxation on the plateau value of methacholine contrac-
tion. Each set of experiments was performed with four tracheas in
parallel. Only one treatment was tested per set. This was the only
approach possible because, with our apparatus, all four tracheas
bathed in the same solution. We also did not want to use these tracheas
over and over again because repeated uses could modify the contrac-
tile and relaxation properties of the specimens. Because all experi-
ments were done with the same strain of normal mice, we believe that
comparison between groups of experiments is valid.

To assess the nicotinic agonist specificity for the receptor, hexa-
methonium (Sigma-Aldrich; 10−2 M), a general nicotinic antagonist,
was added 10 min before the addition of increasing concentrations of
DMPP. The effect of α-bungarotoxin (Sigma-Aldrich; 10−3 M), a
nicotinic antagonist specific for the α7-nAChR subunit, was also
tested in the same way.

To ensure that the nicotinic agonist effect observed was directly
mediated by smooth muscle, epithelium was removed in some experi-
mints by rubbing the luminal area with dental floss (Ultrafloss,
Oral-B). Histological analysis confirmed that this technique success-
fully removed the tracheal epithelium (data not shown). Neuronal
effect was blocked by the administration of the nerve blocker tetro-
dotoxin (TTX; Sigma-Aldrich; 3.1 × 10−6 M) 10 min before addition
of DMPP. NO was also blocked by addition of the NO inhibitor
Nω-nitro-l-arginine methyl ester (l-NAME; Sigma-Aldrich; 10−6 M)
10 min before DMPP.

**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 pro-
teins (RP) (see Table 1). Anti-α3 was raised against a RP correspond-
ing to amino acids 367–502 mapping at the carboxy terminus of
α3-subunit of human origin. Anti-α4 was raised against a RP corres-
ponding to amino acids 342–474 and anti-α7 against a RP corre-
sponding to amino acids 367–502, all mapping at the carboxy termini-
us of their corresponding nAChR subunit of human origin.

**Histological studies.** Tracheal tissue was first fixed in a 4% para-
formaldehyde solution and embedded in paraffin. Ten-micrometer-
thick tissue sections were then sliced and mounted on slides. Hema-
toxylin-eosin staining was performed on slices and studied under light
microscope.

**Isometric studies.** Tracheas denuded from any connective tissue
were placed in Krebs solution (in mM: 118 NaCl, 4.7 KCl, 1.8
KH2PO4, 1.18 MgSO4•7H2O, 2.5 CaCl2•H2O, 25 NaHCO3, and 5.6
D-glucose). Extreme care was taken not to stretch the rings, to keep the
smooth muscle tissue intact. Tracheal rings were mounted isometrically
to force transducers and equilibrated for 1 h in 5-ml organ baths
(Radnoti Four-Unit tissue bath system, Harvard Apparatus) containing
Krebs bicarbonate solution at pH 7.4, maintained at 37°C, and
bubbled with 95% O2-5% CO2. The resting tension was 0.5 g, and the
bath fluid was changed every 15 min. Tissue viability was tested with

The localization of the smooth muscle layer
receptor subunit seen with a red filter shows
the presence of this receptor in the epithelial
(e) layer. D: single staining with α3-subunit
receptor antibody without Evans blue clearly
shows the presence of this receptor subunit in
the epithelial layer.

**Fig. 1.** Detection of α7-subunit protein in mouse tracheal sections by immunofluores-
cence. A: double staining with α3-actin antibody and α3-antibody seen with a green filter
interferes with the α3 staining. B: double staining with α3-actin-Cy3 antibody and α3-receptor
subunit seen with a red filter shows the localization of the smooth muscle layer
(red). C: single staining with α3-receptor subunit (green) with Evans blue (red) shows
the presence of this receptor in the epithelial layer. D: single staining with α3-subunit
receptor antibody without Evans blue clearly shows the presence of this receptor subunit in
the epithelial layer.

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

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Company</th>
<th>Dilution</th>
</tr>
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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>
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<tr>
<td>Alexafluor 488-labeled goat anti-rabbit IgG</td>
<td>Molecular Probes</td>
<td>1:150</td>
</tr>
<tr>
<td>Monoclonal anti-α3-smooth muscle actin, clone 1A4, Cy3 Conjugate</td>
<td>Sigma</td>
<td>1:25</td>
</tr>
</tbody>
</table>

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 (\( 10^{-3} - 10^{-2} \) M) (Fig. 6; \( P < 0.0001, n = 9 \)).

Hexamethonium (\( 10^{-1} \) M) completely inhibited the relaxation induced by DMPP (Fig. 7; \( P = 0.0001, n = 4 \)), 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 \( P = 0.001, n = 6 \)) 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 \(\alpha_3\)-AChRs 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 nAChR agonists. 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).

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 ≤ 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 (●; \(n = 14\)) α-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 (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 ≤ 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 bronchial preparations but relaxed tracheal preparations of the same animal. Given the small size of mice, bronchial strips 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, Takayanagi 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 α3-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 nAChRA could be of interest in the treatment of asthma and other obstructive pulmonary diseases.

GRANTS
This research was funded by Innovatech-Québec.

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 inventors 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


