2-Aminoethoxydiphenyl borate stimulates pulmonary C neurons via the activation of TRPV channels

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Nonmyelinated (C) fibers represent ~75% of the afferent fibers in the vagal branches innervating the entire respiratory tract (2, 14). Cell bodies of these afferent fibers reside in two adjacent but distinct anatomic structures, the nodose and intracranial jugular ganglia. The afferent activity arising from these C fiber endings plays an important role in regulating the respiratory and cardiovascular functions under both normal and abnormal physiological conditions (14, 26). Capsaicin, a pungent active ingredient of hot pepper, is the chemical agent most commonly used in studies of physiological properties and functions of these afferents because of its specificity and potency in stimulating these afferents (18, 19). Bolus injection of capsaicin into the pulmonary circulation stimulates pulmonary C fiber afferents and elicits pulmonary chemoreflex responses (apnea, bradycardia, and hypotension), which are mediated through the TRPV1 activation (14, 26). Indeed, expression of the TRPV1 and other members in the TRPV family in the vagal sensory (nodose and jugular) ganglia has been recently demonstrated (10, 17, 48). However, whether they are expressed by the neurons innervating the lungs is not known. Furthermore, the physiological roles of these TRPV channels, except the TRPV1, in regulating the function of pulmonary afferent nervous system, has been hampered due to a lack of selective activator and blocker of these channels. In light of the ability of 2-APB in activating these TRPV channels (11, 20), this study was therefore carried out: 1) to characterize the cardiorespiratory reflex responses to intravenous (IV) injection of 2-APB in spontaneously breathing rats and to evaluate the role of vagal C fiber afferents in eliciting these responses; 2) to determine the contribution of TRPV1 to the response of single unit pulmonary C fibers to 2-APB in artificially ventilated rats; and 3) to investigate the response of isolated pulmonary capsaicin-sensitive neurons to 2-APB and to differentiate the relative roles of TRPV1 and other TRPV channels in these responses.

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MATERIALS AND METHODS

Animal preparation. The procedures described below were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Sprague-Dawley rats (355–440 g) were initially anesthetized with an intraperitoneal injection of α-chloralose (100 mg/kg; Sigma Chemical, St. Louis, MO) and urethane (500 mg/kg; Sigma) dissolved in a borax solution (2%; Sigma); supplemental doses of α-chloralose (∼10 mg·kg⁻¹·h⁻¹) and urethane (∼50 mg·kg⁻¹·h⁻¹) were IV injected to maintain abolition of pain reflexes elicited by paw pinch. One femoral artery was cannulated for recording the arterial blood pressure (ABP) with a pressure transducer (Statham P23AA). For IV administration of pharmacological agents, the left jugular vein was cannulated, and a catheter was advanced until its tip was positioned just above the right atrium. The volume of each bolus injection was 0.1 ml, which was first injected into the catheter (dead space, ∼0.2 ml) and then flushed into the circulation by an injection of 0.3 ml of saline. A short tracheal cannula was inserted after a tracheotomy, and tracheal pressure (Pₜ) was measured (Validyne MP 45-28) via a side port of the trachea cannula. Body temperature was maintained at ∼36°C by means of a heating pad placed under the animal, which was lying in a supine position. At the end of the experiment, the animal was killed by an IV injection of KCl (200 mg/kg).

Measurements of respiratory and cardiovascular responses. Rats breathed spontaneously via the tracheal cannula. Respiratory flow was measured with a heated pneumotachograph and a differential pressure transducer (Validyne MP45-14) and integrated to give tidal volume (Vₐ). Respiratory frequency (f), inspiratory and expiratory durations (Tᵢ, Tₑ), minute ventilation (Vₑ), heart rate (HR), and ABP were analyzed (Biocybertics TS-100) on a breath-by-breath basis by an online computer.

Recording of pulmonary C fiber activity. Pulmonary C fiber activities were recorded in anesthetized, open-chest, artificially ventilated rats, as described in details previously (18, 27). Briefly, the expiratory outlet of the respirator was placed under a heated trachea above the thoracic inlet. With the head tilted up at 30°, the dietodecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI). Young adult Sprague-Dawley rats (150–200 g) were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and intubated with a polyethylene catheter (PE-150) with its tip positioned in the trachea above the thoracic inlet. With the head tilted up at 30°, DiI was initially dissolved and sonicated in ethanol, diluted in saline (1% ethanol vol/vol), and then instilled into the lungs (0.15 mg/ml; 0.2 ml × 2). After 7–11 days, an interval previously determined to be sufficient for the dye to diffuse to the cell body, the nodose and jugular ganglia were extracted.

Isolation and culture of nodose and jugular ganglion neurons. The animals were anesthetized with halothane inhalation and decapitated. The head was immediately immersed in ice-cold Hanks’ balanced salt solution. Nodose and jugular ganglia were extracted under a dissecting microscope and placed in ice-cold DMEM/F-12 solution. Each ganglion was desheathed, cut into ∼10 pieces, placed in 0.125% type I collagenase, and incubated for 1 h in 5% CO₂ in air at 37°C. The ganglion suspension was centrifuged (150 g, 5 min) and supernatant aspirated. The cell pellet was resuspended in 0.05% trypsin and 0.53 mM EDTA in Hanks’ balanced salt solution for 5 min. The pellet was then resuspended in a modified DMEM/F-12 solution supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 µM MEM nonessential amino acids) and gently triturated with a fine bore fire-polished Pasteur pipette. The dispersed cell suspension was centrifuged (500 g, 8 min) through a layer of 15% bovine serum albumin to separate the cells from the myelin debris. The pellets were resuspended in the modified DMEM/F-12 solution supplemented with 50 ng/ml of 2.5S nerve growth factor, plated onto poly-l-lysine-coated glass coverslips, and then incubated overnight (5% CO₂ in air at 37°C).

Whole cell perforated patch-clamp recording. The methods were described previously (20). Briefly, the coverslip containing the attached cells was placed in the center of a small (0.2 ml) recording chamber that was perfused by gravity feed (VC-6 perfusion valve controller; Warner Instruments, Hamden, CT) with extracellular solution (ECS) and ECS containing capsaizepine or ruthenium red at 2 mM, whereas the chemical stimulants (2-APB and capsaicin) were applied by a pressure-driven drug delivery system (ALA-VMS; ALA Scientific Instruments, Westbury, NY), with its tip positioned to ensure that the cell was fully within the stream of the injectate. The ECS consisted of (in mM) 136 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 0.33 Na₂HPO₄, 10 glucose, and 10 HEPES, pH 7.4. The intracellular solution contained (in mM) 92 potassium gluconate, 40 KCl, 8 NaCl, 0.5 MgCl₂, 10 EGTA, and 10 HEPES, pH 7.2. Recordings were made in the whole cell perforated patch configuration (50 µg/ml of gramicidin) using Axopatch 200B/pCLAMP 9.0 (Axon Instruments, Union City, CA). The experiments were performed at room temperature (22°C). The data were acquired at 5 kHz and filtered at 2 kHz. Series resistance was compensated at ∼80%. The resting membrane potential was held at ∼70 mV.

Experimental design and protocol. Six series of experiments were carried out. Study series 1: the ventilatory and cardiovascular responses elicited by injections of increasing doses of 2-APB (1, 2, and 3 mg/kg) and its vehicle (for the 3 mg/kg dose) were determined. To avoid any accumulated effect, at least 10 min elapsed between two injections in this and subsequent protocols. Study series 2: the role of vagal C fiber afferents in 2-APB-induced cardiorespiratory responses was evaluated. The responses to injections of capsaicin (1 µg/kg) and 2-APB (3 mg/kg) were repeated at 2 and 15 min, respectively, after a bilateral perineural capsaicin treatment (PNCT), which produces a selective and reversible blockade of the C fiber conduction in the vagus nerves (27). Briefly, cotton strips soaked in capsaicin solution (250 µg/ml) were wrapped around a 2- to 3-mm segment of the isolated cervical vagi for 20 min and then removed. The responses to capsaicin and 2-APB were again tested at 60 and 75 min, respectively, after PNCT. Study series 3: single unit pulmonary C fiber responses to
increasing doses of 2-APB (1, 2, and 3 mg/kg) and its vehicle (for the 3 mg/kg dose) were determined in 26 C fibers from 15 rats. Study series 4: this series was carried out to determine the role of the TRPV1 receptor in the 2-APB-induced stimulation of pulmonary C fibers. The 2-APB (3 mg/kg)- and capsaicin (1 μg/kg)-induced afferent responses were studied and compared before and after a constant IV infusion of capsazepine (0.3 mg·kg⁻¹·min⁻¹; 5 min), a selective TRPV1 receptor antagonist (4, 41), or its vehicle. When responses to 2-APB and capsaicin were studied in the same fibers (n = 8), the sequence of the injections of these two chemicals was alternated to achieve a balanced design. Study series 5: the responses to increasing concentrations of 2-APB (30, 100, and 300 μM) or its vehicle (for the 300 μM dose) were determined in 26 C fibers from 15 rats.

Fig. 1. Pulmonary chemoreflex responses to intravenous injection of 2-aminoethoxydiphenyl borate (2-APB) in anesthetized rats are depicted. A–D: experimental records illustrating the responses to intravenous injections of vehicle and 1, 2, and 3 mg/kg of 2-APB, respectively, in an anesthetized rat breathing spontaneously. Injectate (0.1 ml) was first slowly injected into the catheter (dead space, 0.2 ml) and then flushed (at arrow) as a bolus with saline (0.3 ml). Rat body weight, 390 g. Vt, tidal volume; ABP, arterial blood pressure. E: effect of increasing doses of 2-APB on apneic ratio [apneic duration/baseline expiratory duration (Tₑ)]. Apneic duration, longest expiratory duration within 3 s after injection. Baseline Tₑ, average expiratory duration >10 control breaths. Dashed line, apneic ratio of 100% level (no apnea); arrows here and in subsequent figures indicate flush times of the chemicals. *Significantly different (P < 0.05) from the response to vehicle. Data of each group are means ± SE of 12 rats.

Fig. 2. Experimental records are shown illustrating the effect of perineural capsaicin treatment (PNCT) of both cervical vagi on cardiorespiratory responses to injections of capsaicin and 2-APB in an anesthetized rat. A, C, and E: responses to intravenous injections of capsaicin (1 μg/kg) before and 5 and 60 min after PNCT of both vagi, respectively. B, D, and F: responses to injections of 2-APB (3 mg/kg) before and 15 and 75 min after PNCT, respectively. Rat body weight, 365 g.
concentration) were determined in isolated pulmonary capsaicin-sensitive neurons. Study series 6: this series was carried out to differentiate the relative roles of TRPV1 and other TRPV channels in 2-APB-induced inward current in pulmonary capsaicin-sensitive neurons. Responses to 2-APB (300 μM) and capsaicin (0.3 or 1 μM) were studied before and after a 5-min pretreatment with capsazepine (10 μM) or ruthenium red (5 μM); the latter is an inorganic dye and a nonspecific blocker of Ca^{2+} channels and is also known for its nonselective but effective blocking effect on all the TRPV channels (13, 16). The sequence of applications of 2-APB and capsaicin was alternated when they were studied in the same neurons (n = 10).

Materials. A stock solution of 2-APB (100 mM; Tocris, Ellisville, MO) was prepared in DMSO (Sigma) and that of capsaicin (1 mM; Sigma) in 1% Tween 80, 1% ethanol, and 98% saline (study series 1–4) or ECS (study series 5 and 6). Stock solutions of capsazepine (Sigma) and ruthenium red (Sigma) were both prepared in DMSO at a concentration of 30 mM. Solutions of these chemical agents at desired concentrations were then prepared daily by dilution with saline on the basis of the animal’s body weight in study series 1–4 or diluted with ECS in study series 5 and 6.

Statistical analysis. Data were analyzed with a one- or two-way ANOVA. When the ANOVA showed a significant interaction, pairwise comparisons were made with a post hoc analysis (Fisher’s least significant differences). A P value < 0.05 was considered significant. Data are means ± SE.

RESULTS
2-APB-induced pulmonary chemoreflex responses. Bolus injections of 2-APB at the doses of 1 and 2 mg/kg only produced very mild ventilatory and cardiovascular inhibitions. A higher dose (3 mg/kg) of 2-APB consistently elicited apnea, bradycardia, and hypotension (Fig. 1). Both respiratory and cardiovascular responses returned to baseline within 10 s. The reproducibility of the cardiorespiratory responses to this high dose of 2-APB was confirmed in at least three separate experiments. 

Fig. 3. Effect of PNCT of both cervical vagi on ventilatory responses to intravenous injections of capsaicin and 2-APB in anesthetized rats is shown. A and B: ventilatory responses to capsaicin (1 μg/kg) and 2-APB (3 mg/kg), respectively, before (○) and 5 (capsaicin) or 15 (2-APB) min after (*) PNCT of both vagi. f, respiratory frequency; V̇E, minute ventilation. Vertical dashed line depicts time of injection. C and D: apneic responses to capsaicin and 2-APB, respectively. Control (open bars), before PNCT; PNCT (solid bars), 5 (capsaicin) or 15 (2-APB) min after PNCT; recovery (hatched bars), 60 (capsaicin) or 75 (2-APB) min after PNCT. For calculation of apneic ratio, see Fig. 1 legend. Dashed line, apneic ratio of 100% level (no apnea). *Significantly different (P < 0.05) from the corresponding control. Data of each group are means ± SE of 6 rats.
dose of 2-APB was established in four rats, and no tachyphylaxis was observed when at least 10 min elapsed between two injections. The apneic response induced by 2-APB at the dose of 3 mg/kg was clearly shown by a prolonged $T_e$, reaching $545 \pm 73\%$ of the baseline $T_e$ (Fig. 1E). In contrast, the injection of vehicle did not cause any significant change in respiration.

Effect of PNCT on capsaicin- and 2-APB-induced pulmonary chemoreflex responses. In control condition, IV injection of 2-APB (3 mg/kg) induced ventilatory and cardiovascular inhibition that was comparable to the degree and pattern of that caused by 1 $\mu$g/kg of capsaicin (e.g., Fig. 2, A and B). PNCT of both cervical vagi caused only slight but not significant increases in baseline $f$, $V_e$, HR, and ABP. However, it completely abolished the chemoreflexes (apnea, bradycardia, and hypotension) induced by capsaicin (1 $\mu$g/kg), verifying its effectiveness in blocking the conduction of vagal C fiber afferents (Figs. 2 and 3). In the same animals, the 2-APB (3 mg/kg)-induced respiratory and cardiovascular inhibitions were also abolished after the treatment (Figs. 2 and 3). The responses to both capsaicin and 2-APB returned toward the control in $\sim 60$ min; however, the recovery of chemoreflex response to 2-APB was consistently slower and less complete compared with that of capsaicin (e.g., Fig. 2, E and F; Fig. 3, C and D).

Dose dependence of the stimulating effect of 2-APB on pulmonary C fibers. 2-APB stimulated pulmonary C fiber afferents in a dose-dependent manner (Fig. 4). Injections of 2-APB at the dose of 2 mg/kg activated 13 out of 26 C fibers tested, but the average response ($\Delta FA$, 1.13 $\pm$ 0.38 impulses/s; $n = 26$) was not significantly different from that of either 1 mg/kg (0.10 $\pm$ 0.08 impulses/s; $n = 26$) or vehicle (0.09 $\pm$ 0.07 impulses/s; $n = 10$). A higher dose of 2-APB (3 mg/kg) activated all the C fibers tested ($\Delta FA$, 10.82 $\pm$ 2.12 impulses/s; $n = 26$); most of the fibers started to discharge within 1 s, and the activity reached a peak in $\sim 2$ s and returned to baseline within 4 s after the injection. In the majority of the animals, this high dose of 2-APB (3 mg/kg) also elicited a cardiovascular depression; the magnitudes of these responses were comparable to that of 1 $\mu$g/kg of capsaicin (e.g., Fig. 5, A and B).

Effect of capsazepine on capsaicin- and 2-APB-induced pulmonary C fiber activation and cardiovascular responses. After pretreatment with capsazepine, there was no change in the baseline FA of pulmonary C fibers (before capsazepine, 0.01 $\pm$ 0.01 impulses/s, after capsazepine, 0.01 $\pm$ 0.01 impulses/s; $P > 0.05$; $n = 20$). However, the C fiber discharges induced by injection of 1 $\mu$g/kg of capsaicin were almost completely abolished by the treatment ($\Delta FA$: before capsazepine, 12.4 $\pm$ 2.12 impulses/s; after capsazepine, 0.35 $\pm$ 0.13 impulses/s; $P < 0.05$; $n = 11$); so were the capsaicin-induced cardiovascular depressor responses (Figs. 5, 6, and 7); the response to the same dose of capsaicin returned to the control level $\sim 20$ min after capsaicine treatment (10.2 $\pm$ 2.25 impulses/s). In sharp contrast, capsaicine treatment only blocked $\sim 74\%$ of the $\Delta FA$ induced by 2-APB (before capsazepine, 13.4 $\pm$ 1.7 impulses/s, after capsazepine, 3.5 $\pm$ 0.83 impulses/s; $P < 0.05$, $n = 17$), whereas its blocking effect on 2-APB-induced cardiovascular depressor responses was substantially less (Figs. 5, 6, and 7). In a separate group of 16 C fibers, treatment with capsazepine vehicle did not have any detectable effect on the responses induced by either capsaicin (not shown) or 2-APB (Fig. 7A).

2-APB-induced whole cell inward currents in isolated rat pulmonary capsaicin-sensitive neurons. Because of the potency and selectivity of capsaicin in activating the pulmonary C fibers (18), we consider “capsaicin-sensitive neurons” as “C neurons” in this study. To identify the sensory neurons that give rise to pulmonary C fibers for whole cell perforated patch-clamp recording, neurons isolated from nodose and jugular ganglia were selected based on the following criteria: 1) labeled with Dil as indicated by fluorescence intensity; 2) smooth surface and spherical shape without visible processes; 3) whole cell capacitance <30 pF; and 4) responding to capsaicin (1 $\mu$M). Application of 2-APB in the range of 30–300 $\mu$M (4–8 s) evoked incremental whole cell inward currents in a concentration-dependent manner (Fig. 8, A and B). 2-APB (300 $\mu$M) stimulated all the capsaicin-sensitive pulmonary nodose and jugular ganglion neurons held at $-70$
mV; the inward currents evoked by this high concentration of 2-APB and 0.3–1 µM capsaicin followed a very similar pattern and reached a comparable amplitude in 13 out of 24 pulmonary sensory neurons tested, and a clear disparity was displayed in the remaining neurons.

Effect of capsazepine and ruthenium red on capsaicin- and 2-APB-induced whole cell inward currents in isolated rat pulmonary capsaicin-sensitive neurons. Pretreatment with capsazepine (10 µM, 5 min) completely abolished capsaicin (0.3–1 µM, 4–8 s)-induced inward current (Fig. 8, C and D; before capsazepine, 289.5 ± 44.7 pA; after capsazepine, 0.8 ± 0.4 pA; P < 0.05; n = 8) but only inhibited ~60% of the currents induced by 2-APB (300 µM, 4–8 s) (Fig. 8, E and F; before capsazepine, 403.1 ± 74.2 pA; after capsazepine, 186.6 ± 49.8 pA; P < 0.05; n = 9); both capsaicin- and 2-APB-induced inward currents returned to the control level after ~60 min of washout. In contrast, pretreatment with ruthenium red (5 µM, 5 min) almost completely abolished the inward currents induced by both capsaicin (before ruthenium red, 241.7 ± 34.9 pA; after ruthenium red, 0.7 ± 0.3 pA; P < 0.05; n = 6) and 2-APB (before ruthenium red, 293.1 ± 62.3 pA; after ruthenium red, 16.1 ± 4.9 pA; P < 0.05; n = 7); the antagonizing effects of ruthenium red were also reversed after ~60 min of recovery (Fig. 9). Pretreatment with the vehicles of capsazepine and ruthenium red did not have any detectable effect on the inward currents induced by either capsaicin or 2-APB (n = 4).

DISCUSSION

In the present study, we have demonstrated that right atrium injection of 2-APB evoked the classic "pulmonary chemore-

Fig. 6. Effect of pretreatment with capsazepine (CPZ) on averaged pulmonary C fiber and cardiovascular responses to capsaicin and 2-APB in anesthetized and open-chest rats is shown. A and B: response to intravenous injections of capsaicin (1 µg/kg) and 2-APB (3 mg/kg), respectively, before and 2 min after intravenous infusion of capsaicin (0.3 mg·kg⁻¹·min⁻¹; 5 min). ○, before capsaizepine; ●, after capsaizpine; MABP, mean ABP; HR, heart rate. Data are means ± SE of 11 and 17 C fibers in A and B, respectively. Note that capsazepine only slightly shortened but did not prevent the bradycardia induced by 2-APB (B).
coworkers (20) demonstrated that 2-APB at a higher concentration (500 μM) is a common and potent activator of three TRPV channels, TRPV1-3. Using the fluo-4-based Ca^{2+} assay, they further determined that the EC_{50} values of 2-APB were 114 ± 8, 129 ± 13, and 34 ± 12 μM for TRPV1, V2, and V3, respectively, in HEK-293 cells expressing these TRPVs. As the first cloned member of the TRPV subfamily, TRPV1 is a Ca^{2+}-permeable cation channel activated not only by vanilloids, such as capsaicin, but also by endogenous lipids (endocannabinoids, eicosanoids), protons (pH < −5.9), and temperature (>−43°C) (8, 10, 21, 22, 27). The expression of TRPV1 was found exclusively in small- to medium-diameter neurons within dorsal root, trigeminal, and nodose sensory ganglia (8, 10, 25, 41). Both TRPV2 and TRPV3 are insensitive to capsaicin and show a steep dependence of temperature (43). TRPV2 seems to be expressed preferentially in medium-

flex” responses, characterized by apnea, bradycardia, and hypotension. These reflex responses were completely abolished by PNCT of both cervical vagi, suggesting the involvement of vagal C fibers. The single-fiber recording experiments have confirmed that 2-APB (1, 2, and 3 mg/kg) stimulated the individual pulmonary C fibers in a dose-dependent manner. Results obtained from the capsazepine experiment indicated that the stimulatory effect of 2-APB resulted, in part, from the activation of TRPV1. Our patch-clamp studies in isolated pulmonary capsaicin-sensitive neurons have further demonstrated that 2-APB caused a stimulatory effect on these neurons. This stimulating effect was probably mediated through the activation of TRPV1, V2, and V3 channels because it was attenuated but not totally abolished by capsazepine, a selective TRPV1 antagonist (4, 41). In contrast, it was completely abrogated by ruthenium red, a nonselective TRPV channel blocker (13, 16).

2-APB was originally reported as a membrane-permeable antagonist of IP_3 receptors (30) and has been used extensively as an inhibitor of store-operated Ca^{2+} influx and IP_3-mediated Ca^{2+} release (5, 15). Recent studies have revealed a novel effect of 2-APB on TRPV channels (11, 20). In HEK-293 cells transfected with mouse TRPVs, Chung and colleagues (11) reported that 2-APB, at the concentration of 100 μM, generated a robust stimulatory effect on TRPV3, little effect on TRPV1, and no activation of TRPV2. In contrast, Hu and
to large-diameter sensory neurons and is activated by hypoosmolarity (34), growth factor (23), and noxious heat (≥52°C) (9). Expression of TRPV3 has been found not only in sensory neurons (e.g., dorsal root, trigeminal, and superior cervical ganglia) but also in skin, tongue, and other tissues (47). TRPV3 is activated by innocuous temperatures (≥34–38°C) (36, 38, 47) and by the only known chemical stimulus, 2-APB (11, 20). In a recent study using RT-PCR, Zhang and colleagues (48) have demonstrated the presence of temperature-sensitive TRP channel transcripts (TRPVs, TRPN1, and TRPM8) in nodose ganglia gastrointestinal neurons. The expression of TRPV1 and TRPV2 in nodose ganglia was further confirmed by their immunohistochemical staining studies. Whether these TRPV channels are also expressed in the cell bodies of isolated pulmonary neurons and the pulmonary C fiber sensory endings is not known. Based on the results obtained in the present study and the literature described above, we suggest that activation of TRPV1, V2, and V3 is probably involved in the stimulation of these sensory neurons by 2-APB. Our results cannot completely rule out other potential excitatory effects of 2-APB on these neurons. For example, in addition to its inhibitory effect, 2-APB has been shown to activate or potentiate store-operated Ca²⁺ channels and a nonselective cation channel of unknown function (6, 29, 37). However, this possible involvement is less likely in our study because the 2-APB effect was completely abolished by ruthenium red.

Immunohistochemical studies have illustrated the presence of C fiber sensory endings in the mucosa of all sizes of airways in various species, including humans (1, 3, 24). These nerve endings display extensive axon arborization that either extends into the space between epithelial cells or forms network-like plexus immediately beneath the basement membrane of epithelium (1, 3, 24). The superficial locations of these nerve endings suggest an important role of these afferents in regulating the airway responses to inhaled irritants such as sulfur dioxide, ammonia, cigarette smoke, and acid aerosol, etc. Furthermore, pulmonary C fiber afferents are generally known to possess polymodal sensitivity; in addition to chemical irritants (e.g., acid, nicotine, capsaicin), these afferents can also be activated by certain endogenously released autacoids (e.g., prostaglandin, bradykinin, anandamide, etc.) as well as mechanical stimuli such as hyperinflation of the lungs (14, 18, 26, 27, 42). The overall electrophysiological properties and signal transduction processes of pulmonary sensory neurons are regulated by the functional expression of various receptor proteins. Indeed, a number of ligand (e.g., TRPV1, P2X3, purinoceptor, etc.) and voltage-gated ion channels (e.g., tetrodotoxin-resistant Na⁺ channel, Ca²⁺-dependent K⁺ channels, etc.) have been identified on the sensory terminals of C fibers as well as in the isolated C neurons innervating airways and lungs (7, 25). Using 2-APB as a chemical tool and based on the comparison of the antagonistic effects between capsazepine and ruthenium red, we have now for the first time demonstrated the functional expressions of TRPV2 and/or TRPV3 channels in pulmonary C fiber terminals as well as in their cells bodies. Considering the fact that the TRPV channels are the primary sensors of a diverse range of external physical (e.g., heat, cold, mechanical stress) and chemical (e.g., pH, pheromones, osmolarity) stimuli (12, 43), a better understanding of the roles of these channels in regulating the function of airway sensory nerves is certainly very important.

Our present study has provided direct evidence of a distinct stimulatory effect of 2-APB on pulmonary C fiber afferents. Stimulation of these sensory afferents is known to elicit other important defense reflex responses, in addition to the pulmonary chemoreflexes. These reflex responses, such as bronchoconstriction, mucus hypersecretion, airway irritation, and coughing, are mediated through the central nervous system and/or autonomic nervous system (14, 26). Moreover, sensory neuropeptides such as tachykinins (e.g., substance P, neurokinin A) and calcitonin gene-related peptide, synthesized in the cell bodies of pulmonary C fibers in various species, including humans, are released locally from the sensory terminals upon stimulation (28, 39). These peptides are known to act on a number of effector cells (e.g., airway smooth muscles, cholinergic ganglia, inflammatory cells, mucous glands) and produce potent local effects such as bronchoconstriction, plasma extravasation, and edema of airway mucosa (39). Although these reflexes and local responses resulting from the C fiber activation were not measured in this study, 2-APB can, presumably, evoke these airway responses to varying degrees of intensity, depending on the dose administered.

In the present study, we intended to compare the responses induced by 2-APB and capsaicin in the same pulmonary C fibers. Despite the large difference in the doses applied between these two chemicals (3 mg/kg vs. 1 μg/kg), more than half of the C fibers (16 out of 26 fibers tested) displayed an interesting resemblance between the afferent responses to 2-APB and capsaicin. However, certain disparity is evident in the remaining fibers; for example, a greater stimulatory effect of 2-APB than capsaicin was found in six fibers, whereas the
opposite was found in others. This observation also holds true in the responses of isolated pulmonary sensory neurons to these two chemicals. Although the exact underlying mechanism is not known, we suspect that different expressions and distributions of various TRPV channel proteins among pulmonary C fiber terminals should, at least in part, account for their different sensitivities to capsaicin and 2-APB. This explanation is supported by the fact that the difference in the chemoreflex responses to these two chemicals at the same doses was much less (Fig. 3, C and D) because these reflex responses reflect integrated activities of the entire population of pulmonary C fibers. In addition, we have demonstrated that PNCT completely abolished the cardiorespiratory responses to both capsaicin and 2-APB (Figs. 2 and 3), indicating that these responses were indeed mediated through pulmonary C fibers.

In sharp contrast to the responses to capsaicin, the cardiovascular depressor responses (bradycardia and hypotension) induced by 2-APB persisted even after the pretreatment with capsazepine (e.g., Fig. 6B). This observation lends additional support to our conclusion that the pulmonary chemoreflex responses elicited by 2-APB did not result exclusively from activation of the TRPV1 channel.

In summary, this study using 2-APB as a chemical tool to activate TRPV1, V2, and V3 channels has suggested the functional expression of these channels in pulmonary C neurons. This conclusion is further supported by our observation that the 2-APB-evoked response is only partially blocked by capsazepine but completely abrogated by ruthenium red. The conclusion that the pulmonary chemoreflex responses elicited by 2-APB did not result exclusively from activation of the TRPV1 channel.

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