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Dopamine type 2 receptor expression and function in rodent sensory neurons projecting to the airways

Christian Peiser,[1] Marcello Trevisani,[2] David A. Groneberg,[1] Q. Thai Dinh,[1] Doerthe Lencer,[1] Silvia Amadesi,[2] Barbara Maggiore,[2] Selena Harrison,[2] Pierangelo Geppetti,[2] and Axel Fischer[1]

[1]*Department of Pediatric Pneumology and Immunology, Charité - Virchow Campus, Berlin, Germany; and* [2]*Department of Experimental and Clinical Medicine, Pharmacology Unit, University of Ferrara, Italy*

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Peiser, Christian, Marcello Trevisani, David A. Groneberg, Q. Thai Dinh, Doerthe Lencer, Silvia Amadesi, Barbara Maggiore, Selena Harrison, Pierangelo Geppetti, and Axel Fischer. Dopamine type 2 receptor expression and function in rodent sensory neurons projecting to the airways. *Am J Physiol Lung Cell Mol Physiol* 289: L153–L158, 2005. First published March 25, 2005; doi:10.1152/ajplung.00222.2004.—Agonists of the dopamine receptors have been demonstrated to have bronchodilatory properties in pathologically constricted airways. The mechanism by which these agonists induce bronchodilatation is thought to involve airway sensory nerves. In this study, the expression and function of dopamine D_2 receptor were examined in sensory ganglia supplying the airways. Neuronal dopamine D_2 receptor mRNA expression was demonstrated by single-cell RT-PCR following laser-assisted microdissection. The projection of the neurons to the airways was confirmed by retrograde neuronal labeling. In functional studies, dopamine D_2 receptor agonists (AR-C65116AB and ropinirole) inhibited intraneuronal calcium mobilization in rat capsaicin-sensitive primary sensory neurons and capsaicin-induced plasma extravasation in the rat trachea. Our results provide support to the hypothesis that dopamine D_2 receptor activation inhibits neurogenic inflammation and proinflammatory reflex responses.

D_2 dopamine receptor; jugular-nodose ganglion; dorsal root ganglion; rat

PHARMACOLOGICAL EVIDENCE SUGGESTS that for the treatment of diseases associated with airway hyperreactivity and airway obstruction, such as bronchial asthma or chronic obstructive pulmonary disease, administration of inhaled dopamine induces bronchodilatation (5, 23) in a dose-related manner (21). Furthermore, in the therapy of airway diseases, dual dopamine D_2 receptors (D_2)/β_2-receptor agonists have been shown to have beneficial effects compared with conventional β_2-receptor agonists alone (4). Functional experiments indicate that this additional effect is mediated through normalizing the pathological increased activity of sensory nerves (17).

For a better understanding of these clinical observations, more knowledge is needed about the dopamine system in the respiratory tract, especially concerning sensory nerves projecting to the airways. Dopamine has been demonstrated to be localized in some peripheral tissues, also in several nonneuronal cell types (8). There is a 4.4-fold increase in dopamine content compared with the local noradrenalin concentration (1). This excess of dopamine suggests that it acts in a role other than a noradrenaline precursor (20). Various studies suggest that dopamine is released from nerve endings in the periphery and interacts with specific receptors. D_2, which are divided into two different isoforms derived from alternative RNA splicing (13, 25), are present on many neurons in the periphery (24), and their activation can modulate neuronal activity (26). So far, expression of D_2 mRNA has been only reported in rat sensory ganglia (33).

However, to our knowledge, no information is available on the expression of dopamine receptors in sensory neurons projecting into the respiratory system, thus the aim of this study is to determine expression and function of dopamine receptors, with particular respect to D_2.

MATERIALS AND METHODS

Animals. For the experiments described below, male Sprague-Dawley rats (Charles River) were used. All animal studies carried out are conform to Institutional Animal Care and Use Committee guidelines.

Retrograde neuronal tracing. Rats were anesthetized by intramuscular injection of ketamine hydrochloride (Ketanest, Parke-Davis) followed by xylazine hydrochloride (Rompun, Bayer). The trachea was exposed and incised between two bridges of cartilage. A microsyringe was inserted into the right principal bronchus, and the fluorescent tracer Fast Blue (Dr. K. Illing, Gross-Umstadt, Germany) as a 2% aqueous solution containing 1% DMSO was injected. The incision sites were covered with fibrin glue to avoid tracer diffusion. (For more details see Ref. 9.) Animals were killed 7 days later, and ganglia were removed for immunohistochemistry.

Tissue preparation. For tissue preparation, the animals were anesthetized by an overdose of pentobarbital sodium and killed by exsanguination. Ganglia were excised and either fixed in Zamboni's solution for immunohistochemistry, quick-frozen in liquid nitrogen for mRNA extraction, quick-frozen in melting isopentane for laser-assisted cell picking, or placed in cold PBS for cultivation.

Single cell isolation. For laser-assisted cell isolation, serial cryosections (6 μm) were collected on coverslips, stained 2 min with hemalaun, dipped in deionized water, and transferred to 100% ethanol. After cells of interest were selected under an inverted microscope (Axiovert 135, Zeiss), adjacent cells were photolyzed by laser microbeam (337-nm wavelength, PALM) via the epifluorescence illumination path. A sterile needle guided by a micromanipulator was used for picking the isolated cells via adhesive forces and for subsequent transfer into a reaction tube for RT-PCR.

Immunohistochemistry. Tissue sections, fixed in Zamboni's solution, were cut on a cryostat at 8 μm and air-dried for 30 min. The sections were incubated with a blocking solution containing 1% bovine serum albumin (BSA) and 10% normal swine serum in 0.1 M phosphate buffer for 60 min, followed by a rabbit polyclonal antiserum to rat D_2 receptor (Biotrend) in a 1:1,000 dilution. Sections

Address for reprint requests and other correspondence: C. Peiser, Charité-Virchow Campus, Dept. of Pediatrics, Biomedical Research Center, Augustenburger Platz 1, 13353 Berlin, Germany (E-mail: christian.peiser@charite.de).

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were rinsed with phosphate buffer and incubated in FITC-conjugated anti-rabbit IgG from sheep (Amersham) in a 1:200 dilution. Preabsorption with the soluble antigen was carried out to demonstrate the specificity of the immunofluorescence.

RNA extraction. Total RNA was extracted from ganglia from adult rats (200–250 g) using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. Quality and quantity of RNA were controlled by optical density (OD) 260/280 nm and agarose gel electrophoresis. For the extraction of RNA from isolated cells, the cell sections were lysed in 10 μl of first-strand buffer: 1% Igepal CA-630 (Sigma) and 4% RNase inhibitor (Perkin-Elmer) in 52 mM Tris·HCl (pH 8.3), 78 mM KCl, and 3.1 mM $MgCl_2$.

Reverse transcription. For first-strand cDNA synthesis, 2 μg of total RNA were denaturated with 1 μg of random hexamer (50 μM, Roche) for 10 min at 70°C, followed by an incubation on ice for 1 min. Reverse transcription was carried out in a mixture containing 4 μl of 5× first-strand buffer (Life Technologies), 2 μl DTT (100 mM, Life Technologies), 5 μl dNTP mix (2 mM, MPI Fermentas), and 1 μl SuperScript II-reverse transcriptase (Life Technologies). Samples were incubated at 20°C for 10 min and at 42°C for 60 min. The reaction was stopped by heating to 70°C for 15 min.

Polymerase chain reaction. PCR was undertaken using primers specific for both the long and short isoforms of D_2. Primers (Tib Molbiol) for the detection of the rat D_2 were as follows: forward primer, 5′-CAGCAGTCGAGCTTTCAGAG-3′, reverse primer, 5′-CTGGTGCTTGACAGCATCTC-3′. For PCR, 2 μl cDNA, 5 μl 10× buffer (Perkin-Elmer), 7.5 μl dNTP mix (2 mM, MPI Fermentas), 2 μl of each primer, and 0.5 μl *Taq* DNA polymerase (5 U/μl) (Perkin-Elmer) were supplemented with distilled water to a final volume of 50 μl. DNA was amplified using the following conditions: 3 min, 94°C; 35 cycles, 30 s, 94°C; 45 s, 55°C and 1 min, 72°C; and 1 min, 72°C. (For more details see Ref. 27.) The same protocol was followed for the detection of the D_2 mRNA in single cells except that the number of cycles was increased to 60 cycles. Amplification products were visualized via gel electrophoresis.

Sequencing. Sequencing of the PCR products to verify amplification specificity was carried out according to the manufacturer's protocol (ABI Prism sequencing protocol) using the Ampli-Taq FS Big Dye Terminator (Perkin-Elmer).

Isolation and cultivation of thoracic dorsal root ganglion neurons. Thoracic dorsal root ganglion (DRG) neurons dissected from newborn rats (2–3 days old) were removed and rapidly placed in cold PBS before being transferred to collagenase/dispase (1 mg/ml dissolved in Ca^{2+}-Mg^{2+}-free PBS) (Roche) for 35 min at 37°C. Enrichment of the fraction of nociceptive neurons was obtained following the methods reported previously (12). After the enzymatic treatment, ganglia were rinsed three times with Ca^{2+}-Mg^{2+}-free PBS and then placed in 2 ml of cold DMEM (GIBCO) supplemented with 10% fetal bovine serum (heat inactivated, GIBCO), 2 mM L-glutamine (GIBCO), 100 U/ml penicillin (GIBCO), and 100 μg/ml streptomycin (GIBCO). The ganglia were then dissociated into single cells by several passages through a series of syringe needles (23-gauge needle down to 25-gauge needle). Finally, the complex of medium and ganglia cells was centrifuged (200 *g* for 5 min). The cell pellet was resuspended in DMEM medium [supplemented with 100 ng/ml of mouse nerve growth factor (mouse-NGF-7S, Roche) and 2.5 μM cytosine-β-D-arabinofuranoside free base (ARA-C, Sigma)]. Cells were plated on poly-L-lysine (8.3 μM, Sigma)- and laminin (5 μM, Sigma)-coated, 25-mm glass coverslips and kept for 2 to 5 days at 37°C in a humidified incubator gassed with 5% CO_2 and air.

Table 1. *Fast Blue-labeled neurons*

Fast Blue-Labeled Neurons	<25 nm	≥25 nm
42.5% D_2 receptor immunoreactive	31.7%	68.3%
57.5% D_2 receptor not immunoreactive	34.7%	65.3%

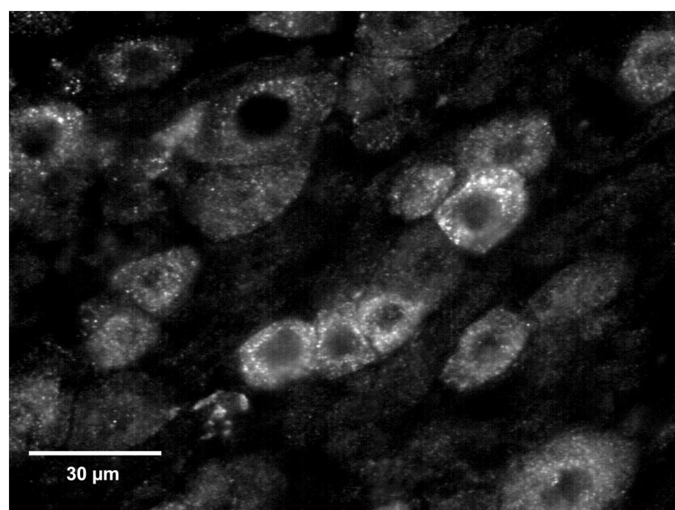

Fig. 1. Immunohistochemical staining for D_2 dopamine receptors in jugular-nodose ganglia of the rat is shown.

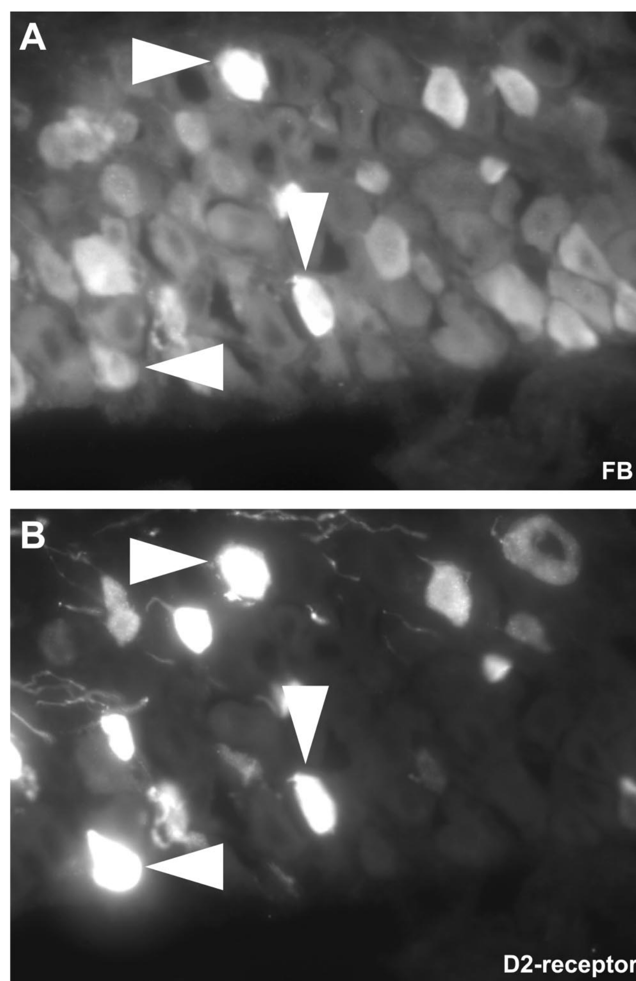

Fig. 2. Shown is retrograde neuronal tracing from the respiratory tract: neurons from the jugular-nodose ganglia stained with the fluorescent marker Fast Blue (FB) taken up from lung projections. A substantial number of the FB-labeled neurons (arrowheads, *A*) were D_2 dopamine receptor immunoreactive (arrowheads, *B*).

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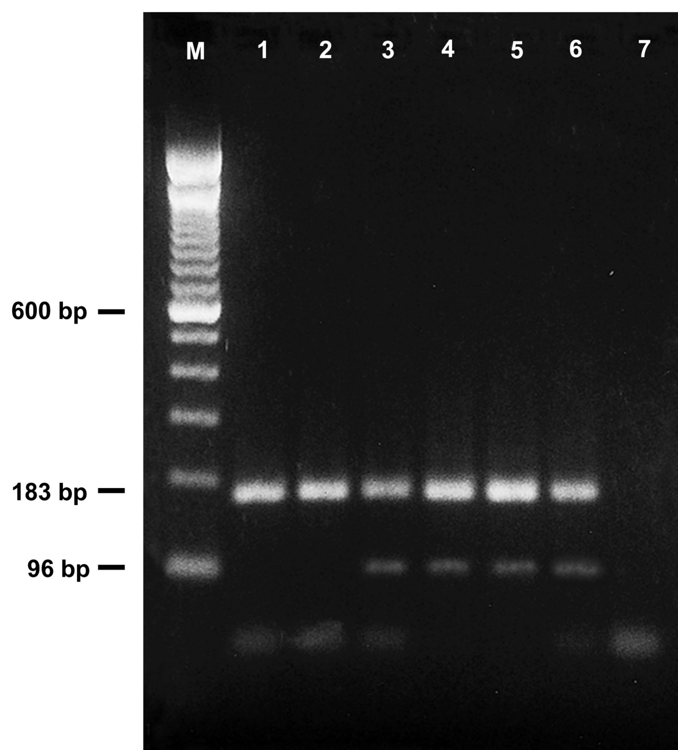

Fig. 3. RT-PCR amplification bands using primers against the long (183 bp) and short (96 bp) isoform of the D_2 dopamine receptor in rat ganglia. M, marker; *lane 1*, principal bronchus; *lane 2*, lung hilus; *lane 3*, peripheral lung; *lane 4*, cervical dorsal root ganglion; *lane 5*, thoracic dorsal root ganglion; *lane 6*, jugular-nodose ganglion; *lane 7*, negative control.

Measurement of intracellular Ca^{2+} concentrations in cell culture. Plated neurons were loaded with fura-2 AM (dissolved in DMSO, 3 μM, Societá Italiana Chimici) in Ca^{2+} buffer solution (of the following composition: 1.4 mM $CaCl_2$, 5.4 mM KCl, 0.4 mM $MgSO_4$, 135 mM NaCl, 5 mM D-glucose, and 10 mM HEPES with 0.1% BSA, pH 7.4, for 40 min at 37°C), washed twice with the Ca^{2+} buffer solution, and transferred to a chamber on the stage of a Nikon Eclipse TE300 microscope. The dye was excited at 340 and 380 nm to indicate relative intracellular Ca^{2+} changes by the F_{340}/F_{380} ratio recorded with a dynamic image analysis system (Laboratory Automation 2.0; RCS, Florence, Italy). Intracellular Ca^{2+} mobilization was monitored following two electrical field stimulations (EFS; 10 Hz, 40 mA, 1 ms, 10 s) 20 min apart. The D_2 receptor agonist ropinirole (7) (1 μM) or its vehicles was added to the chamber. In a second set of experiments, the D_2 receptor antagonist sulpiride (10) (10 μM) was added to the chamber 15 min before ropinirole (supplied by AstraZeneca UK). At the end of any of the experiments, neurons were challenged with the transient receptor potential vanilloid type 1 agonist capsaicin (1 μM; Sigma).

Plasma extravasation studies. Rats (250–300 g) were anesthetized with pentobarbital sodium. Evans blue (EB, 30 mg/kg) was injected into the jugular vein 1 min before an intravenous injection of capsaicin (10 μg/kg, Sigma) or substance P (10 nmol/kg, Sigma). The animals were killed 5 min after injection of the stimuli. Pretreatments with the D_2 receptor agonists AR-C65116AB (30 μg/kg iv, supplied by AstraZeneca UK) and ropinirole (100 μg/kg iv, supplied by AstraZeneca UK) or their respective vehicles were performed 15 min before the injection of the dye. In a second set of experiments, animals were pretreated with the D_2 receptor antagonist sulpiride (1 mg/kg iv) 20 min before the injections of the D_2 agonists. The trachea was removed, cleaned, weighed, and incubated in 1 ml of formamide for 24 h in the dark at room temperature. The amount of the extravasated EB was measured spectrophotometrically at 620 nm.

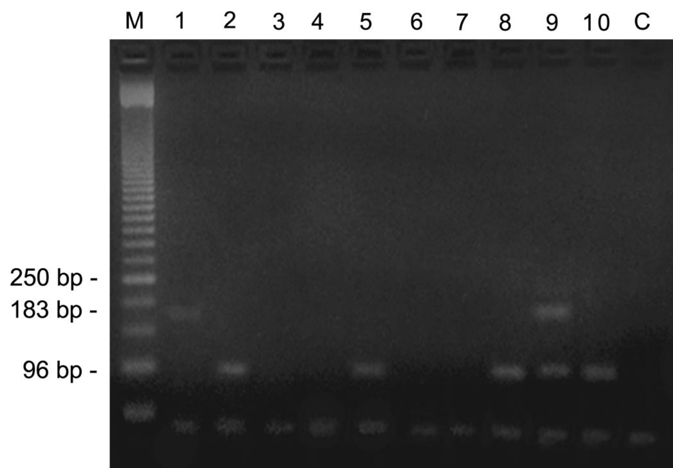

Fig. 4. Single-cell RT-PCR amplification bands using primers against the long (183 bp) and short (96 bp) isoform of the D_2 dopamine receptor in 10 neuronal sections of the jugular-nodose ganglion complex. C, negative control.

RESULTS

To investigate the expression of D_2 in jugular-nodose complex and DRG of the rat on the protein level, immunohistochemistry was carried out using an antibody that detects both the long and the short splice form of D_2. Some of the neurons of jugular-nodose ganglia (42.5%) were immunoreactive for D_2, comprising neurons with small (<25 nm) and large (≥25 nm) diameters of perikarya (Fig. 1, Table 1). Immunohistochemistry in DRG revealed identical results (data not shown). Preabsorption of the antiserum with the corresponding antigen resulted in absence of labeling.

Because the neurons of the jugular-nodose ganglion complex provide afferent innervation to a number of thoracic and abdominal organs, retrograde neuronal tracing studies were performed to identify the neurons that project to the airways. The analysis of the ganglia that were collected 7 days after tracer injection into the main bronchus showed a substantial number of Fast Blue-labeled neurons (Fig. 2) that are immunoreactive for D_2.

The identification of D_2 at the mRNA level was done via RT-PCR from the total RNA of jugular-nodose ganglia. The

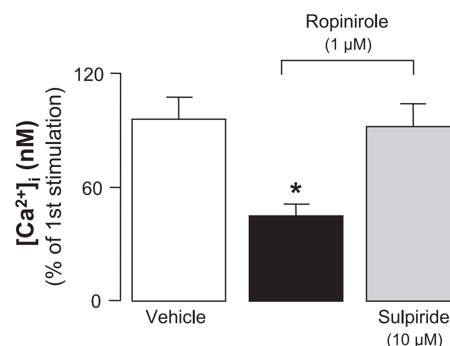

Fig. 5. Electrical field stimulation (EFS)-induced intracellular Ca^{2+} mobilization in cultured rat dorsal root ganglion neurons in the absence (open column) or presence (filled column) of ropinirole or sulpiride (gray column). The dopamine D_2 receptor agonist ropinirole (1 μM) significantly (* $P < 0.05$) reduced the 2nd EFS-induced response (42% ± 13 of the 1st EFS). Pretreatment with sulpiride (10 μM) reversed the inhibitory effect produced by ropinirole (81% ± 5 of the 1st EFS).

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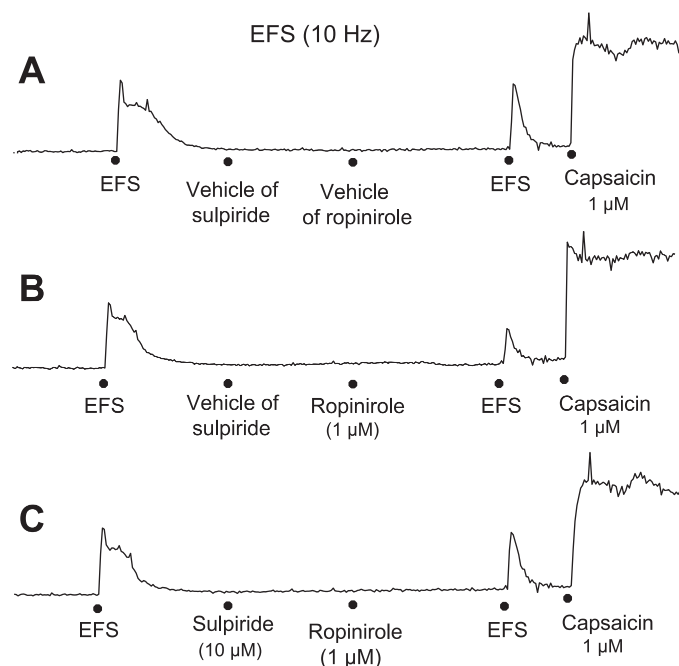

Fig. 6. Typical tracing of intracellular Ca^{2+} mobilization in cultured rat dorsal root ganglion neurons. *A*: 1st EFS-induced Ca^{2+} mobilization was similar to the 2nd EFS when applied after treatment with the vehicles for sulpiride and ropinirole. *B*: incubation with ropinirole (1 μM) significantly attenuated the second EFS-induced Ca^{2+} mobilization. *C*: sulpiride (10 μM) blocked the inhibitory effect of ropinirole (1 μM).

primer pair used for amplification detects both the long and the short isoforms that lead to products with different length. In jugular-nodose ganglia, DRG, principal bronchus, lung hilus, and peripheral lung, D_2 expression was found (Fig. 3). No DNA was detected in samples run without RNA or RT, respectively. Amplification specificity was verified by sequencing of the PCR products.

To ensure that the amplified D_2 transcripts were of neuronal origin, neurons of the jugular-nodose ganglion complex were identified microscopically, isolated using a laser-assisted cell picking device, and harvested for RT-PCR. The analysis of splicing variants at the level of single sensory neurons revealed that ~60% of the sensory neurons express either isoform of D_2 mRNA. The short isoform was found to be expressed more frequently than the long one. Occasionally, neurons were found to express both splice forms of D_2 transcript in a single cell (Fig. 4).

The effect of D_2 receptor activation was studied in capsaicin-sensitive cultured rat DRG neurons. The basal concentration of intracellular Ca^{2+} (50.0 ± 7 nM, $n = 98$) was significantly increase following the first EFS application (191.5 ± 8.2 nM, $n = 98$). The application of the second EFS induced a Ca^{2+} mobilization (186.3 ± 7.8 nM, $n = 98$) that was similar to that of the first (97% of the first EFS). Ropinirole produced a statistically significant reduction (107.2 ± 6.1 nM, $n = 29$, 58% of inhibition, $P < 0.05$) of the EFS-induced intracellular Ca^{2+} mobilization compared with the effect observed in the presence of its vehicle. This inhibitory effect was prevented by the pretreatment with sulpiride (126.6 ± 7.7 nM, $n = 24$; Fig. 5). Capsaicin (1 μM) was applied at the end of each experiment to select only the primary sensory neurons (Fig. 6).

In another experimental setup, the capsaicin- and substance P-induced plasma extravasation in the trachea of adult rats was measured. The ability of the two D_2 receptor agonists AR-C65116AB (30 μg/kg iv) and ropinirole (100 μg/kg iv) to block plasma extravasation and the reversibility of this effect by the D_2 receptor antagonist sulpiride (1 mg/kg iv) were tested. Capsaicin (10 μg/kg iv) was able to induce plasma extravasation in rat trachea (16.6 ± 1.1 ng of EB/mg tissue) compared with the basal plasma extravasation level (8.9 ± 0.6 ng of EB/mg tissue). The plasma extravasation induced by capsaicin was reduced by both AR-C65116AB (11.9 ± 0.8 ng of EB/mg tissue, 61% inhibition) and ropinirole (12.8 ± 0.5 ng of EB/mg tissue, 49% inhibition). The inhibitory effects induced by AR-C65116AB and ropinirole were prevented by pretreatment with sulpiride (1 mg/kg; 16.1 ± 1 ng of EB/mg tissue and 15.7 ± 0.8 ng of EB/mg tissue, respectively). On the other hand, AR-C65116AB (22.4 ± 1.4 ng of EB/mg tissue) and ropinirole (18.9 ± 1.2 ng of EB/mg tissue) did not significantly modify the substance P-induced plasma protein extravasation (21.6 ± 2.3 ng of EB/mg tissue) in the rat trachea (Fig. 7).

DISCUSSION

Whereas numerous publications deal with D_2 in the central and peripheral nerve system, there are fewer data concerning its expression and function in sensory neurons projecting to the airways. Because pharmacological and clinical data have shown that the inhalative application of dopamine or D_2 agonists have a bronchodilatory effect in cases of bronchial obstruction (4, 5, 23) with a dose-related (21) reduction of breathlessness, cough, and sputum production (15, 16), we have focused the role of D_2 on the respiratory system.

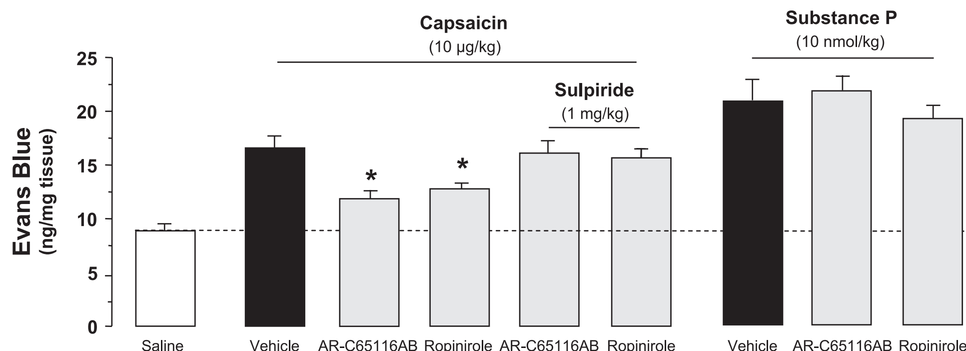

Fig. 7. Capsaicin- and substance P-induced plasma extravasation in rat trachea. The increase in plasma extravasation induced by capsaicin was significantly (**P* < 0.05) reduced by AR-C65116AB (30 μg/kg, 61% inhibition) and ropinirole (100 μg/kg, 49% inhibition). The inhibition of plasma protein extravasation induced by these 2 dopamine D_2 receptor agonists was prevented by the addition of sulpiride (1 mg/kg). Neither AR-C65116AB nor ropinirole did significantly modify the amount of extravasated dye induced by substance P. Each entry is means ± SE of at least 6 experiments.

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In the present study, we have investigated jugular-nodose ganglia and DRG of the rat. We have found that D_2 is expressed in these ganglia at the mRNA level (RT-PCR) and protein level (immunohistochemistry). In addition, we could show that the amplified D_2 receptor transcripts are of neuronal origin (single-cell PCR) and that many of the immunoreactive neurons project to the airways (retrograde neuronal tracing). In another experimental setup, capsaicin-induced plasma extravasation, a standardized method of quantifying the consequences of sensory nerve activation in the trachea (11), was measured; D_2 agonists were able to reduce capsaicin-induced plasma extravasation without affecting plasma extravasation induced by intravenous administration of substance P. These findings indicate a prejunctional side of action of the D_2 agonist. Next, we have provided evidence that agonism on D_2 receptor inhibits EFS-induced intracellular Ca^{2+} mobilization in capsaicin-sensitive primary cultured cells prepared from newborn rats and hence excitation in primary sensory neurons. Because these neurons activate proinflammatory reflex responses and neurogenic inflammation, D_2 agonists may exert anti-inflammatory effects in a number of organs, including the airways. In addition, stimulation of D_2 by the dopamine agonists AR-C651116AB and ropinirole inhibits plasma extravasation in the trachea of adult rats as an important marker for a neuroinflammatory tissue reaction.

In previous studies, the effects of dopamine in the cardiovascular system have been reported (22, 30, 31). Stimulation of D_2-like dopamine receptors, which mainly have a prejunctional localization (19), mediate arterial vasorelaxation indirectly by decreasing the sympathetic vasoconstrictor tone (6, 14). But there are only a few reports about the action of dopamine on the respiratory system. In cases of bronchoconstriction, the administration of dopamine, systemically or topically, has a bronchodilatatory effect. Michoud et al. (23) measured the effect of increasing doses of dopamine (infused or inhaled) on pulmonary resistance in asthmatic and healthy subjects; dopamine significantly decreased histamine-induced bronchoconstriction in both groups. Kamikawa and Shimo (18) have demonstrated, in an in vitro experiment, that dopamine inhibits cholinergically mediated contractions in guinea pig isolated bronchial muscle. Weyman-Jones et al. (32) have shown in the rat that D_2 agonists inhibit neuropeptide release from sensory nerves projecting to the airways. Cabezas et al. (5) have studied patients with crisis of bronchial asthma, patients with bronchial hyperreagibility without acute exacerbation, and healthy subjects; dopamine inhalation induced bronchodilatation in patients with peracute asthma crisis, determined by measurement of a significant increase of forced expiratory volume at the first second and of forced vital capacity. Clinical studies using a dual D_2/β_2-receptor agonist, applied via inhalation in patients with chronic obstructive pulmonary disease, have confirmed a reduction of breathlessness, cough, and sputum production (15, 16), an effect that was dose related (21). Jackson and Simpson (17) have shown that dopamine, given as an infusion into dogs, affected the ability of rapidly adapting receptors to respond to histamine by an action on D_2. Recently, it has been shown that dopamine and D_2 agonists inhibit vagal sensory nerve-induced microvascular leakage in the rat; this effect could be blocked by the antagonist sulpiride (3).

The airways are densely innervated by sensory and autonomic nerve fibers, and a common feature of inflammation is an increase in nerve density (28) and an enhancement of sensory nerve activity (2). The antiobstructive effect of dopamine or its agonists is mediated through normalizing the pathologically increased activity in sensory nerves (26). The expression of dopamine receptor transcripts has been detected in rat sensory and sympathetic ganglia so far. Van Dijken et al. (29) have determined the distribution of D_2 in rat spinal cord that provides anatomical support for the involvement of D_2 in modulating autonomic control. Xie et al. (33) have demonstrated the presence of mRNAs for both isoforms of D_2, in cervical sympathetic ganglia and DRG. This suggests that D_2 may transduct distinct intracellular signals in these tissues. However, the expression of dopamine receptors in sensory neurons projecting to the respiratory system is a novel finding.

In conclusion, the results reported in the presented study demonstrate the presence of D_2 on rat airway afferent neurons at both transcriptional and translational levels. Furthermore, we have shown functional modulations due to D_2 agonists like inhibition of EFS-induced intracellular Ca^{2+} mobilization and capsaicin-induced plasma extravasation.

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DISCLOSURES

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