Am J Physiol Lung Cell Mol Physiol 287: L272–L278, 2004.
First published April 9, 2004; 10.1152/ajplung.00393.2003.

Functional TRPV4 channels are expressed in human airway smooth muscle cells

Yanlin Jia, Xin Wang, LoriAnn Varty, Charles A. Rizzo, Richard Yang, Craig C. Correll, P. Tara Phelps, Robert W. Egan, and John A. Hey

Neurobiology, Schering-Plough Research Institute, Kenilworth, New Jersey 07033

Submitted 17 November 2003; accepted in final form 5 April 2004

Jia, Yanlin, Xin Wang, LoriAnn Varty, Charles A. Rizzo, Richard Yang, Craig C. Correll, P. Tara Phelps, Robert W. Egan, and John A. Hey. Functional TRPV4 channels are expressed in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 287: L272–L278, 2004. First published April 9, 2004; 10.1152/ajplung.00393.2003.—Hypotonic stimulation induces airway constriction in normal and asthmatic airways. However, the osmolarity sensor in the airway has not been characterized. TRPV4 (also known as VR-OAC, VRL-2, TRP12, OTRPC4), an osmotic-sensitive cation channel in the transient receptor potential (TRP) channel family, was recently cloned. In the present study, we show that TRPV4 mRNA was expressed in cultured human airway smooth muscle cells as analyzed by RT-PCR. Hypotonic stimulation induced Ca^{2+} influx in human airway smooth muscle cells in an osmolarity-dependent manner, consistent with the reported biological activity of TRPV4 in transfected cells. In cultured muscle cells, 4α-phorbol 12,13-didecanoate (4-αPDD), a TRPV4 ligand, increased intracellular Ca^{2+} level only when Ca^{2+} was present in the extracellular solution. The 4-αPDD-induced Ca^{2+} response was inhibited by ruthenium red (1 μM), a known TRPV4 inhibitor, but not by capsazepine (1 μM), a TRPV1 antagonist, indicating that 4-αPDD-induced Ca^{2+} response is mediated by TRPV4. Verapamil (10 μM), an L-type voltage-gated Ca^{2+} channel inhibitor, had no effect on the 4-αPDD-induced Ca^{2+} response, excluding the involvement of L-type Ca^{2+} channels. Furthermore, hypotonic stimulation elicited smooth muscle contraction through a mechanism dependent on membrane Ca^{2+} channels in both isolated human and guinea pig airways. Hypotonicity-induced airway contraction was not inhibited by the L-type Ca^{2+} channel inhibitor nifedipine (1 μM) or by the TRPV1 inhibitor capsazepine (1 μM). We conclude that functional TRPV4 is expressed in human airway smooth muscle cells and may act as an osmolarity sensor in the airway.

calcium influx; fluorometric imaging plate reader; hypotonic solution; smooth muscle contraction; vanilloid receptor-related osmotically activated channel

AIRWAY SMOOTH MUSCLE CONTRACTION in response to various endogenous mediators is an important cause for airway narrowing in asthma. The contraction of airway smooth muscle is initiated by an increase in the free Ca^{2+} in the cytoplasm. Airway smooth muscle contraction generally involves a combination of both Ca^{2+} entry through membrane channels and Ca^{2+} release from intracellular stores (i.e., sarcoplasmic reticulum). Several membrane Ca^{2+} channels have been detected on airway smooth muscle cells and contribute to Ca^{2+} increase during contraction. These channels include voltage-operated Ca^{2+} channels, receptor-operated Ca^{2+} channels, and store-operated Ca^{2+} channels.

TRPV4, a Ca^{2+}-permeable cation channel in the transient receptor potential (TRP) channel family, has been cloned from the rat, mouse, human, and chicken (24, 34). The TRPV4 channel is widely expressed in mammalian tissues, including lung, heart, kidney, sensory neurons, sympathetic nerves, brain, skin, gut, salivary gland, sweat glands, inner ear, endothelium, and fat tissue (14, 15, 24, 29, 34, 41). TRPV4 was also detected by RT-PCR in a human bronchial epithelial cell line (10). The expression of TRPV4 in airway smooth muscle has not been studied. Heterologously expressed TRPV4 channel can be activated by hypotonic solutions (24), phorbol derivatives (12, 38), heat (14, 40), and several endogenous substances, including arachidonic acid and anandamide (30, 39). These activating stimuli open TRPV4 channels through distinct mechanisms (37). Activation of TRPV4 channel increases intracellular Ca^{2+} concentration and thus may play an important role in Ca^{2+} signaling during osmotic stimulation. An abnormality of the osmotic regulation has been observed in mice lacking TRPV4, suggesting that it may subserve a sensor function for osmolarity change (25). In mice lacking TRPV4, a reduced sensitivity to pressure and acidic nociception has also been reported (35). In addition, TRPV4 plays a role in hypotonic stimulus-induced pain in rats, conformed by antisense-induced decrease in expression of the channel (1). The physiological role of TRPV4 activation may vary among different tissues. A recent study showed that hypotonic stimulation activates maxi K^+ channels and regulatory volume decrease response in human bronchial epithelial cells through a Ca^{2+}-dependent mechanism. Because the cells also express TRPV4, it is possible that TRPV4 may be involved in hypotonic solution-induced regulatory volume decrease response through the maxi K^+ channel (10). The direct evidence for the physiological role of TRPV4 channel in native tissues has not been reported.

It has been shown that decreasing osmolarity of the airways by inhaling hypotonic aerosols is a potent stimulus for airway narrowing in subjects with asthma (2, 3, 31). Hypotonic solution also contracts isolated human airways (11, 20). However, the mechanism for hypotonic stimulation-induced airway contraction is not clear. Whether an osmolarity sensor is expressed in the airways and involved in the regulation of airway tone is not known.

In the present study, we investigated the expression of TRPV4 in cultured human airway smooth muscle cells and characterized the channel function of native TRPV4 in these cells. The functional role of TRPV4 in the regulation of airway

Address for reprint requests and other correspondence: Y. Jia, Neurobiology, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033 (E-mail: yanlin.jia@spcorp.com).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

 1040-0605/04 $5.00 Copyright © 2004 the American Physiological Society

Downloaded from http://ajplung.physiology.org/ by 10.220.33.2 on October 13, 2017

smooth muscle tone was also investigated in isolated human and guinea pig airways.

MATERIALS AND METHODS

The present studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and the Animal Welfare Act in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited program.

Materials. Capsaicin, capsazepine, carbachol, DNase IV, collagenase (type IA), and ruthenium red were purchased from Sigma Chemical (St. Louis, MO). Hanks' balanced salt solution (HBSS) was obtained from GIBCO. Verapamil and nifedipine were purchased from ICN Biomedicals (Aurora, OH). Indomethacin, CP-99994, and SR-48968 were synthesized by Chemical Research, Schering-Plough Research Institute (Kenilworth, NJ).

Human airway smooth muscle cells. Primary cultured human bronchial smooth muscle cells (HBSMC) were purchased from Bio Whittaker (Walkersville, MD) and maintained in smooth muscle cell growth media (SmGM-2, Bio Whittaker) at 37°C in humidified air containing 5% CO_2. HBSMC were subcultured into black-walled, clear-based 96-well plates, and confluent cells from the 4th to 7th passages were used for intracellular Ca^{2+} measurement.

RT-PCR. Poly(A) RNA was isolated from cultured HBSMC using the MicroPoly(A) Pure RNA Isolation kit (Ambion). RT-PCR was performed using One-Step RT-PCR (GIBCO). Primers: TRPV1: 5′-CAGGCTCTATGATCGCAGGA, 3′-CGTGTAGCTGGCGTTGACA; TRPV4: 5′-GAGCAATGGCCGCAACGA, 3′-GCCGTGTGTCCTCATCCGTC0; TRPV2: 5′-GAGCAATGGCCGCAACGA, 3′-GCCGTGTGTCCTCATCCGTC. Glyceraldehyde-3-phosphate dehydrogenase primers (Clontech) were used as a positive control. The resulting PCR products for TRPV4 and TRPV2 were subcloned and sequenced for verification of TRPV amplification.

Measurements of intracellular Ca^{2+} concentration using the fluorometric imaging plate reader. Intracellular Ca^{2+} concentration in cultured HBSMC was measured using the fluorometric imaging plate reader (FLIPR) technique as described previously (17, 18). Briefly, cells were incubated with the Ca^{2+}-sensitive fluorescence dye fluo 4-AM (5 μg/ml; Molecular Probes, Eugene, OR) in HBSS (GIBCO) containing 0.4% BSA for 45 min at 37°C. The dye-loading solution was removed, and the cells were washed three times with HBSS containing 0.4% BSA (osmolarity = 310 osmoles per liter). The plates were then placed into a FLIPR (Molecular Devices). Intracellular Ca^{2+} concentration was estimated by fluorescence change detected by FLIPR. Hypotonic buffer of varying strength was prepared according to Liedtke et al. (24). Responses to hypotonicity (90–70% osmolarity of isotonic solution, or 279–217 osmoles per liter) or 4α-phorbol 12,13-didecanoate (4α-PDD) were elicited by direct additions to an individual culture well during real-time recording. The responses were tested in both Ca^{2+}-containing and Ca^{2+}-free conditions. In Ca^{2+}-containing conditions, the Ca^{2+} concentration in all the hypotonic and isotonic buffers was held constant.

Airway tissue isolation from humans and guinea pigs. The role of TRPV4 in the regulation of airway smooth muscle tone was investigated in isolated human airways. Guinea pig airway shares many physiological similarities with the human airway and is more available than human tissues. Therefore, isolated guinea pig trachealis was used to further characterize the hypotonic stimulation-induced airway smooth muscle contraction.

Human airways. Bronchial rings were isolated from human donor lungs procured by Tissue Transformation Technologies (Edison, NJ). The tissues used were from one male and three females (20–56 yrs old) without diagnosed airway diseases. Isometric force was measured on bronchial rings cryopreserved and thawed as previously described with brief modifications (Ref. 33, the cryofreezing tissues were equilibrated at 4°C for 10 min before transfer to −80°C).

Guinea pig airways. Male Hartley guinea pigs (400–700 g; Charles River, Bloomington, MA) were killed with CO_2 and their tracheae were immediately excised and cut into rings for the isometric force measurements. In some experiments, the epithelial layer of the trachea was removed by passing a cotton gauze strip through the tracheal tube as described before (19). Only two adjacent rings located immediately above the carina were used to measure the contraction responses.

Isometric force measurement. Isolated airway rings from humans and guinea pigs were mounted on hooks, connected to force transducers (FT03; Grass, Quincy, MA), and incubated in Krebs buffer containing (in mM): 118 NaCl, 2.55 $CaCl_2$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 24.9 $NaHCO_3$, 11.1 glucose, and 2 μM indomethacin (pH 7.4). The solution was bubbled with 95% O_2-5% CO_2 in 25-ml organ baths at 37°C. The passive tension was set at 1 g, and the tissue was equilibrated for 60 min. Tracheal rings were then preincubated with or without tested compounds. Isometric force of airway rings in response to different stimulators was recorded. In guinea pig airways, maximal contraction induced by KCl (80 mM) or carbachol (10 μM, see Fig. 5*B*) was recorded at the end of the experiments. Airway contraction in response to stimulators was expressed as percentage of KCl-induced maximal contraction. Hypotonic buffer was made according to Liedtke et al. (24). The responses were tested in both Ca^{2+}-containing and Ca^{2+}-free conditions. In Ca^{2+}-containing conditions, the Ca^{2+} concentration in all the hypotonic and isotonic buffers was held constant.

Data analysis. All results are expressed as means ± SE. A one-way ANOVA was performed on the different treatment groups to determine significant effects of the treatments. Post hoc analysis among the different groups was performed with a Dunnett's *t*-test. Student's *t*-test was used when only two groups were compared. A value of $P < 0.05$ was accepted as the level of statistical significance.

RESULTS

TRPV4 expression in human airway smooth muscle cells. To determine the expression of TRPV4 in HBSMC, we performed RT-PCR on RNA isolated from primary cultured cells using oligos designed against TRPV4 and various other known cation channels in the TRPV family. In the course of our studies, we identified two members of the TRPV subfamily of TRP channel receptors that were expressed at the level of mRNA. One of these cation channels was TRPV4 (Fig. 1), whereas the other was TRPV2 (also known as VRL1 channel, Fig. 1). Using oligos designed against TRPV1 (also known as type 1 vanilloid receptor or VR1), we established that the TRPV1 was not present in the airway smooth muscle cells (Fig. 1). Subsequent sequencing of the RT-PCR products confirmed identification of the mRNA of TRPV4 and TRPV2. A duplicate reaction done in the absence of reverse transcriptase yielded no products.

TRPV4 channel function in cultured HBSMC. Activation of TRPV4 induces Ca^{2+} influx through the channel. The function of TRPV4 was characterized in primary cultured human airway smooth muscle cells. Intracellular Ca^{2+} level in response to hypotonicity in cultured cells was measured using the FLIPR technique. Hypotonic stimulation (90–70% of isotonic solutions) increased intracellular Ca^{2+} concentration in an osmolarity-dependent manner in cultured HBSMC (Fig. 2). The Ca^{2+} response to hypotonic buffer was observed only when Ca^{2+} was present in the extracellular buffer, indicating a Ca^{2+} influx through a membrane ion channel (Fig. 2). Capsaicin (10 μM), a TRPV1 agonist, had no effect on intracellular Ca^{2+} in the cell (data not shown).

Downloaded from http://ajplung.physiology.org/ by 10.220.33.2 on October 13, 2017

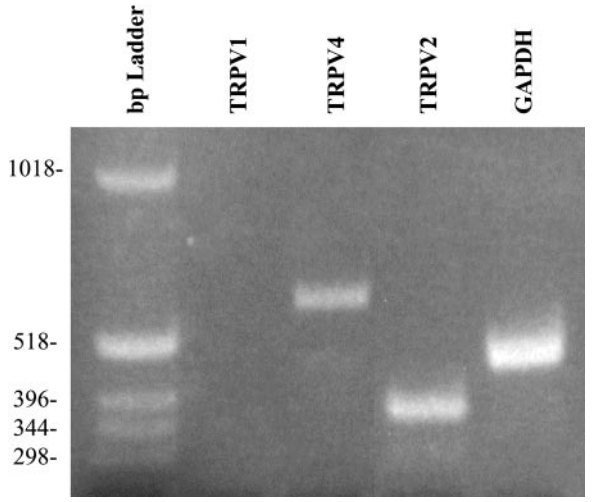

Fig. 1. TRPV4 is expressed in human airway smooth muscle cells. RT-PCR was performed using mRNA isolated from primary human bronchial smooth muscle cells (HBSMC). Oligos designed against human TRPV4, TRPV1, and TRPV2 were used to determine qualitative expression of these highly related genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as positive control. Results indicate that whereas mRNA for TRPV4 and TRPV2 is expressed, TRPV1 is not expressed in HBSMC. TRP, transient receptor potential.

TRPV4 is also activated by the phorbol derivative 4α-PDD (38). In the present study, 4α-PDD also induced Ca^{2+} influx in cultured HBSMC only when Ca^{2+} was present in the extracellular solution (Fig. 3*A*) but not in Ca^{2+}-free conditions (Fig. 3*B*). When cells were pretreated with ruthenium red (1 μM, 10 min), a TRPV4 blocker (36, 38), the 4α-PDD-induced Ca^{2+} response was inhibited by 75%. On the contrary, the 4α-PDD-induced Ca^{2+} response was not inhibited by capsazepine, a TRPV1 channel inhibitor, or by verapamil, an L-type voltage-gated Ca^{2+} channel (VGCC) inhibitor (Fig. 3*B*).

Hypotonic stimulation-induced airway contraction in isolated human bronchus. Airway smooth muscle contraction in response to hypotonic buffer was tested in cryopreserved bronchus isolated from human lung tissues. Isometric force was measured in response to hypotonic solution. As indicated in Fig. 4*A*, hypotonic buffer induced smooth muscle contraction in human bronchus in the presence of Ca^{2+}. When Ca^{2+} was removed from the buffer, hypotonicity-induced airway smooth muscle contraction was significantly decreased (Fig. 4*A*, $P < 0.05$).

Hypotonic stimulation-induced airway contraction in isolated guinea pig trachealis. Isolated guinea pig trachealis was used to further characterize the hypotonic stimulation-induced airway smooth muscle contraction. As in human airways, hypotonic buffer also induced contraction of isolated guinea pig trachealis in an osmolarity-dependent manner in the presence of Ca^{2+} (Fig. 4*B*). Removal of airway epithelium did not change the contraction induced by hypotonic stimulation in guinea pig trachealis (Fig. 4*B*, $P = 0.34$). Hypotonic stimulation-induced airway contraction was abolished in the Ca^{2+}-free condition and was restored by adding Ca^{2+} to the buffer (Fig. 4*C*). On the other hand, carbachol-induced contraction is decreased, but not abolished, in the Ca^{2+}-free condition (Fig. 4*C*).

Pharmacological regulation of the hypotonicity-induced airway contraction was tested next, using guinea pig trachealis. Nifedipine (1 μM), an L-type Ca^{2+} channel inhibitor, had no effect on hypotonicity-induced airway contraction (Fig. 5*B*), excluding the involvement of L-type Ca^{2+} channels. Similarly, capsazepine (1 μM), a TRPV1 channel antagonist, did not affect hypotonic stimulation-induced airway contraction (Fig. 5*B*). Capsazepine is also known to inhibit VGCC in rat dorsal root ganglion neurons with $IC_{50} = 7.7$ μM (7). To exclude its effect on VGCC in the airways, we tested the effect of capsazepine on KCl-induced contraction. KCl is known to depolarize smooth muscle cells and induce airway contraction through a VGCC-dependent mechanism. KCl-induced airway contraction can only be inhibited by nifedipine, an L-type VGCC inhibitor, but not by capsazepine (10 μM, results not shown), indicating that capsazepine has no effect on VGCC in the guinea pig airways and can be used as a TRPV1 inhibitor in the tissue. The observation that capsazepine has no effect on

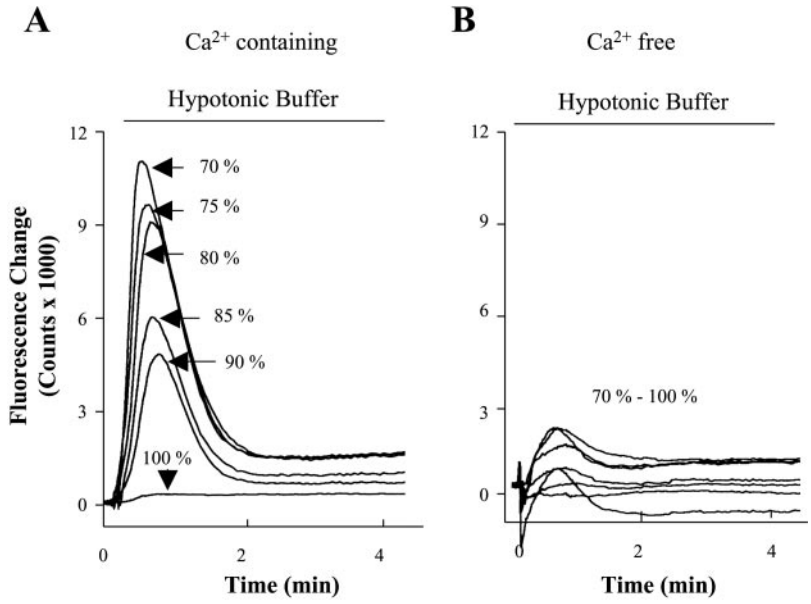

Fig. 2. Hypotonic stimulation induced Ca^{2+} influx in cultured HBSMC. Intracellular Ca^{2+} was determined by fluo 4 fluorescence change using a fluorometric imaging plate reader. Representative traces show the intracellular Ca^{2+} responses to increasing hypotonicity (90–70% of isotonic solution) in Ca^{2+}-containing (*A*) and Ca^{2+}-free (*B*) conditions. The traces represent mean values of triplicate determinations. The experiments were repeated 3 times in triplicate.

Downloaded from http://ajplung.physiology.org/ by 10.220.33.2 on October 13, 2017

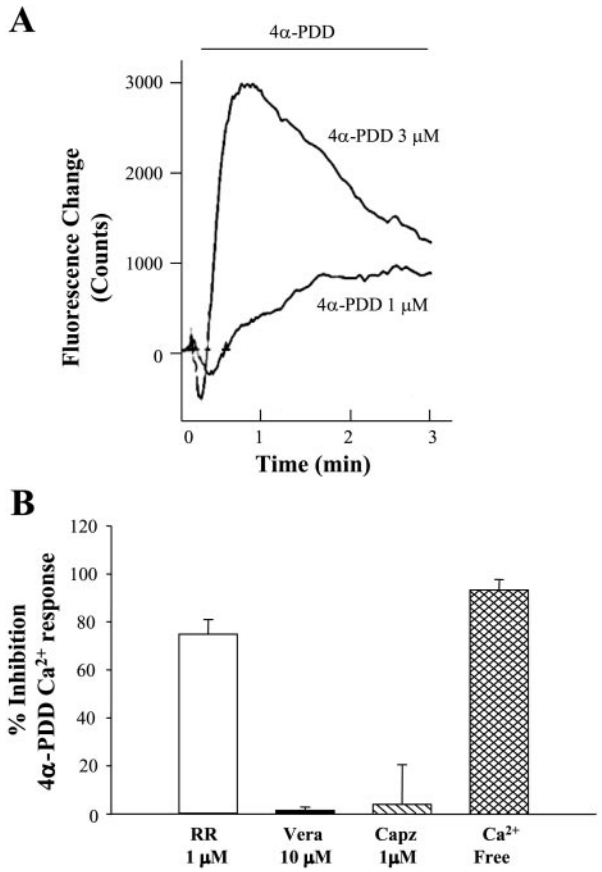

Fig. 3. 4α-Phorbol 12,13-didecanoate (4α-PDD) induced Ca^{2+} response in cultured HBSMC. *A*: representative traces show that 4α-PDD increased intracellular Ca^{2+}. Traces represent mean values of triplicate determinations. The experiments were repeated 3 times. *B*: effect of Ca^{2+} channel inhibitors on 4α-PDD-induced Ca^{2+} response in HBSMC. Cells were preincubated with or without various Ca^{2+} channel inhibitors for 10 min. Ca^{2+} levels in response to 4α-PDD (3 μM) were recorded. The response in a Ca^{2+}-free condition was also recorded. Results are means ± SE, $n = 3$–5. RR, ruthenium red; Vera, verapamil; Capz, capsazepine.

hypotonic stimulation-induced airway contraction indicates that TRPV1 is not involved in the contractile response.

TRPV4 channels are also expressed in sensory neurons (24). Activation of airway sensory nerve releases neuropeptides and induces airway smooth muscle contraction through neurokinin (NK) receptors. To exclude the involvement of tachykinin-containing airway sensory nerves in hypotonicity-induced airway contraction, we also evaluated the effect of specific NK receptor antagonists on hypotonic stimulation-induced airway smooth muscle contraction. Capsaicin is known to activate airway sensory nerves and induces airway smooth muscle contraction through NK receptors (26). CP-99994 (0.3 μM), an NK1 receptor antagonist (27), and SR-48968 (0.3 μM), an NK2 receptor antagonist (27), induced significant inhibition on capsaicin-induced airway contraction, indicating that these concentrations of antagonists effectively inhibit NK receptors in isolated guinea pig airways (Fig. 5*C*). On the contrary, the same concentrations of CP-99994 and SR-48968 had no effect on hypotonicity-induced airway contraction (Fig. 5*C*), excluding the involvement of airway sensory nerves in hypotonicity-induced airway contraction.

We also tested the effect of ruthenium red on smooth muscle contraction. Ruthenium red (10^{-6} M) increased carbachol-induced airway contraction in isolated guinea pig trachealis, indicating a nonspecific effect on smooth muscle contraction (results not shown). For this reason, the effect of ruthenium red on hypotonic stimulation-induced airway contraction is not tested.

DISCUSSION

TRPV4 is an osmolarity-sensitive cation channel, which has been previously identified in the lung using the Northern blot method (24) and in an airway epithelial cell line by RT-PCR (10). In the present study, we showed that TRPV4 mRNA is

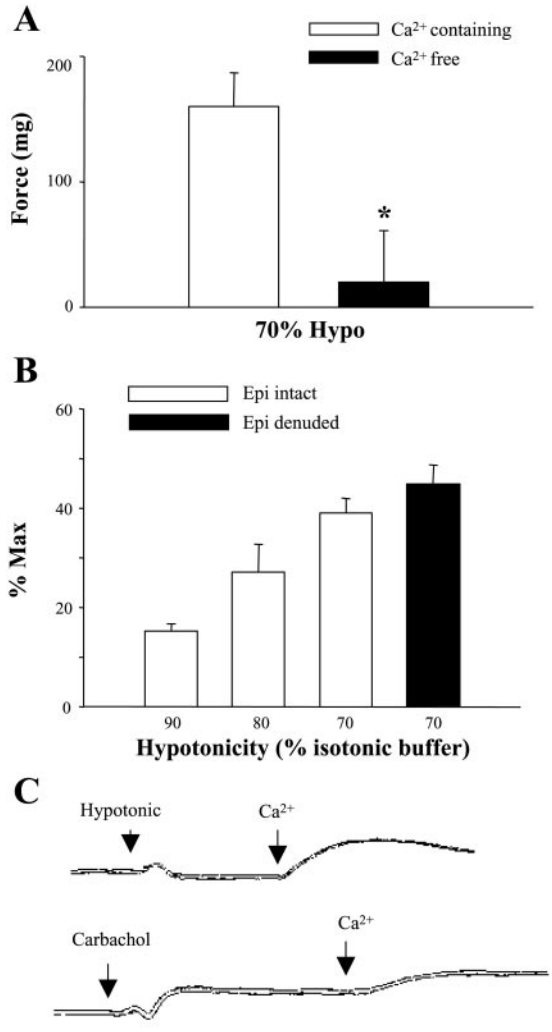

Fig. 4. Hypotonic stimulation induces airway contraction in isolated human and guinea pig airways. *A*: airway contraction in cryopreserved human bronchus in response to hypotonic (Hypo) stimulation (70% of isotonic solution) under Ca^{2+}-containing and Ca^{2+}-free conditions ($n = 5$). *B*: isometric force induced by hypotonic solution was recorded on epithelium (Epi)-intact (open bars, $n = 4$) and denuded (filled bar, $n = 8$) trachealis from guinea pigs. %Max, percentage of maximal contraction to KCl (80 mM). *C*: representative traces showed the isometric force induced by hypotonic stimulation (70% of isotonic buffer) or carbachol (10 μM) in guinea pig trachealis in the absence and presence of Ca^{2+}. The recording started in Ca^{2+}-free conditions. Ca^{2+} was added as indicated. The experiments were repeated 3 times. Data are means ± SE. $^*P < 0.05$ compared with Ca^{2+}-containing group.

Downloaded from http://ajplung.physiology.org/ by 10.220.33.2 on October 13, 2017

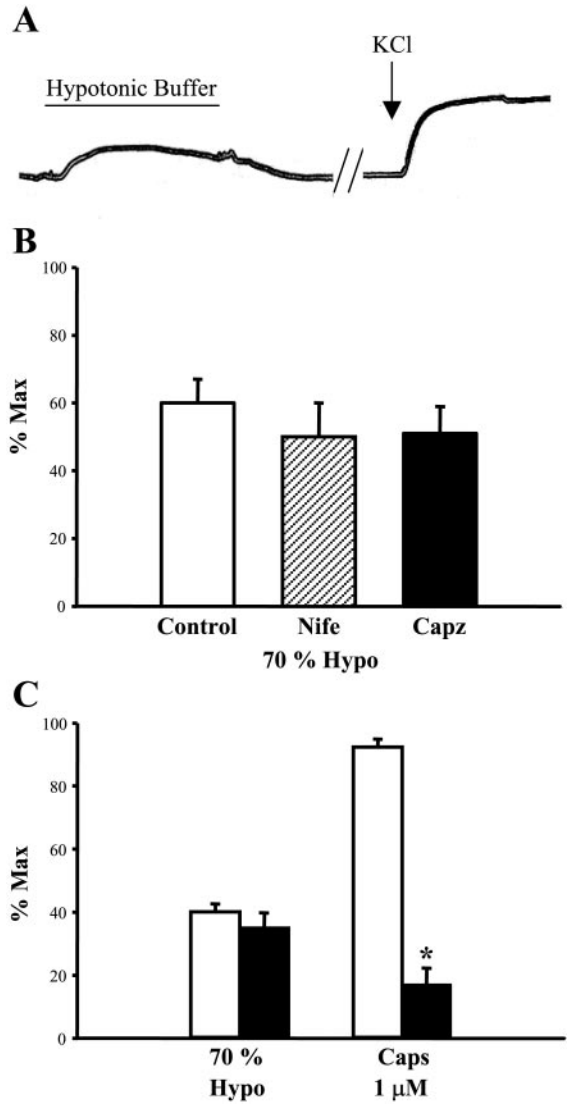

Fig. 5. Regulation of hypotonicity-induced airway contraction in isolated guinea pig trachealis. *A*: representative traces show the hypotonic stimulation (70%)-induced airway contraction. Maximal contraction in response to KCl (80 mM) was recorded at the end of the experiment. *B*: effects of an L-type Ca^{2+} channel blocker and a TRPV1 channel blocker on hypotonicity-induced airway contraction. Trachealis was pretreated with or without nifedipine (Nife; 1 μM) or capsazepine (Capz; 1 μM) for 30 min. Airway contraction in response to 70% hypotonic solution was recorded. *C*: effects of neurokinin receptor antagonists on hypotonicity-induced airway contraction. Trachealis was pretreated with (filled bar, $n = 8$) or without (open bar, $n = 8$) CP-99994 (0.3 μM) and SR-48968 (0.3 μM) for 90 min before addition of the stimulators. $*P < 0.05$ compared with capsaicin control group. Hypo, hypotonic buffer; Caps, capsaicin.

also expressed in HBSMC. We also characterized TRPV4 channel function in human airway smooth muscle cells. This is the first study to demonstrate the functional expression of an osmolarity sensor in airway smooth muscle cells.

TRPV4 is a Ca^{2+}-permeable cation channel. We evaluated its function by measuring intracellular Ca^{2+} level in response to hypotonic stimulation and ligand-mediated receptor activation. Hypotonic solution and 4α-PDD, a TRPV4 activator (38), increased intracellular Ca^{2+} concentration in cultured HBSMC. The Ca^{2+} responses to a range of hypotonic solutions (90–70%) in HBSMC are similar to the Ca^{2+} changes in response to hypotonic stimulation (97–75%) in the chicken TRPV4-transfected Chinese hamster ovary cells reported by Liedtke et al. (24). The Ca^{2+} responses were dependent on the presence of extracellular Ca^{2+}, indicating that Ca^{2+} influx through membrane channels, but not Ca^{2+} release from intracellular store, predominates in the response. Moreover, the 4α-PDD-induced Ca^{2+} response was inhibited by the TRPV4 channel blocker ruthenium red (36, 38). These observations are consistent with the TRPV4 channel function in airway smooth muscle cells.

We also conducted experiments to exclude the role of several other TRPV and Ca^{2+} channels in the Ca^{2+} response. It is unlikely that TRPV1 channels are involved in the Ca^{2+} response in airway smooth muscle cells because *1*) TRPV1 mRNA is not expressed in the cell, *2*) the TRPV1 agonist capsaicin did not induce Ca^{2+} influx into the cells (data not shown), and *3*) capsazepine, a TRPV1 antagonist, had no effect on the 4α-PDD-induced Ca^{2+} response. The L-type VGCC is functionally expressed in airway smooth muscle cells (13, 16). This channel seems not be involved in the Ca^{2+} response because verapamil, an L-type VGCC inhibitor, did not inhibit the 4α-PDD-induced Ca^{2+} response in airway smooth muscle cells. TRPV2 is also expressed in human airway smooth muscle cells at the mRNA level as shown in Fig. 1. It is unlikely that the TRPV2 channel is involved because the Ca^{2+} response is activated by 4α-PDD and hypotonic stimulation, whereas the TRPV2 channel can be activated only by heat (>52°C, 4). We cannot exclude the TRPV2 completely because no specific TRPV2 antagonist is available. TRPV5 and TRPV6 are unique members of the TRPV channels. Their molecular sequence is 74% identical to each other but reveal only 22–24% homology with other members of the TRPV family, including TRPV4 (30). They also express unique channel characteristics that distinguish them from other TRPV channels. For example, unlike other TRPV channels, TRPV5 and TRPV6 are constitutively active, whereas other TRPV channels are activated by stimuli such as ligands (TRPV1), heat (TRPV1, TRPV2, TRPV4), protons (TRPV1), and hypotonic stimulation (TRPV4). Because TRPV5 and TRPV6 are less identical to TRPV4, both structurally and functionally, we did not test their expression and function in the present TRPV4 study. It is unlikely that TRPV5 and TRPV6 are involved in the observed Ca^{2+} response in airway smooth muscle cells because the Ca^{2+} response we observed was activated by hypotonic stimulation and 4α-PDD, whereas TRPV5 and TRPV6 are constitutively active. Together, these results suggest that TRPV4 channels are functionally expressed in human airway smooth muscle cells. The presently characterized TRPV4 channel may be a new addition to the list of membrane Ca^{2+} channels on airway smooth muscle cells.

It has been reported that hypotonic stimulation induces airway smooth muscle contraction in human airways in vivo and in vitro (2, 3, 11, 20, 31). In the present studies, we show that hypotonic solution-induced airway contraction is dependent on the extracellular Ca^{2+} in both human and guinea pig airways. In guinea pig airways, hypotonicity-induced smooth muscle contraction is abolished when Ca^{2+} is removed from organ bath solution and contraction is recovered by addition of Ca^{2+} to the solution. In contrast, carbachol-induced airway contraction (initiated by Ca^{2+} release from sarcoplasmic reticulum) is not abolished in the absence of external Ca^{2+}. These

Downloaded from http://ajplung.physiology.org/ by 10.220.33.2 on October 13, 2017

results suggest that hypotonicity-induced airway smooth muscle contraction is initiated through the activation of membrane Ca^{2+} channels but not by Ca^{2+} release from intracellular stores.

Among the few Ca^{2+} channels that have been detected on the airway smooth muscle cell membrane, the newly detected TRPV4 channel appears to be the only channel that can be activated by hypotonic stimulation. Therefore, TRPV4 is the most plausible channel involved in hypotonic stimulation-induced airway contraction. However, the L-type VGCC is also expressed on the airway smooth muscle cell membrane. Nifedipine, an L-type VGCC inhibitor, is known to inhibit VGCC in airway smooth muscle cells (22). Nifedipine did not inhibit the hypotonicity-induced airway smooth muscle contraction, indicating that the L-type VGCC is not involved in the contraction. The TRPV1 inhibitor capsazepine did not inhibit hypotonicity-induced smooth muscle contraction either, excluding the role of TRPV1 channels in the contraction. Although it is most likely that the TRPV4 channel is activated and involved in hypotonicity-induced airway smooth muscle contraction, we could not confirm this notion using the TRPV4 inhibitor ruthenium red because ruthenium red also increases smooth muscle contraction by inhibition of myosin light chain phosphatase (42). In the present study, we tested the effect of ruthenium red on airway smooth muscle contraction. Indeed, ruthenium red increases carbachol-induced airway contraction, indicating a nonspecific effect on smooth muscle tone. Therefore, ruthenium red is not a suitable pharmacological tool in studies involving smooth muscle contractility.

TRPV4 is also expressed in sensory neurons (24). It is known that activation of sensory nerves in the isolated airways induces tachykinin release, resulting in smooth muscle contraction through NK receptors. To exclude the possibility that hypotonic stimulation may induce airway smooth muscle contraction through the activation of TRPV4 on airway sensory nerves, the effects of NK receptor NK1 and NK2 antagonists were tested. Inhibition of NK1 and NK2 receptors did not affect the contraction of guinea pig trachealis induced by hypotonicity, indicating that tachykinin-containing sensory nerves are most likely not involved in hypotonicity-induced airway smooth muscle contraction. TRPV4 is also expressed in a human airway epithelium cell line (10). Removal of airway epithelium from guinea pig trachealis did not inhibit the contraction induced by hypotonicity, indicating a contractile mechanism independent of airway epithelium. Therefore, it is likely that hypotonicity-induced airway contraction is through a direct action on airway smooth muscles.

The physiological role of TRPV4 in airway function remains to be elucidated. It has been reported that the osmolarity of airway surface fluid is ~80% of isotonic body fluids (222 vs. 285 osmoles per liter) in normal subjects (21). The osmolarity of airway surface fluid is significantly decreased in some airway disease conditions such as asthma (21). Airway surface fluid is also hypotonic in rat and mouse (5, 6). This level of hypotonicity is likely to be sufficient to activate TRPV4 if airway smooth muscle cells are exposed in situ. In normal airways, the tight junction in airway epithelium serves as a barrier between airway surface fluid and smooth muscles. Therefore, the airway smooth muscle is not exposed to hypotonic airway surface fluid, and TRPV4 on smooth muscle cells may not be activated. However, when airway epithelium is disrupted in inflammatory airway diseases such as asthma (8, 23), airway smooth muscle may be exposed to the hypotonic airway surface fluid, and TRPV4 on the cell may be activated. Indeed, inhaling hypotonic aerosols is a potent stimulus for airway narrowing in subjects with asthma (2, 3, 31). Inhalation of distilled water or hypotonic saline induces a fall in forced expiratory volume in 1 s (FEV1) in asthma patients but not in normal subjects (33).

In summary, we have presently demonstrated that TRPV4 is functionally expressed in cultured human airway smooth muscle cells. Activation of TRPV4 on airway smooth muscle cells increases intracellular Ca^{2+} level, which may induce smooth muscle contraction. The role of TRPV4 in hypotonicity-induced airway constriction in normal and diseased human airways merits further investigation.

ACKNOWLEDGMENTS

We thank Drs. Luquan Wang, Peng Wang, and Motasim Billah for helpful scientific discussions.

REFERENCES

1. **Alessandri-Haber N, Yeh JJ, Boyd AE, Parada CA, Chen X, Reichling DB, and Levine JD.** Hypotonicity induces TRPV4-mediated nociception in rat. *Neuron* 39: 497–511, 2003.
2. **Allegra L and Bianco S.** Nonspecific bronchoreactivity obtained with an ultrasonic aerosol of distilled water. *Eur J Respir Dis* 61: 41–49, 1980.
3. **Anderson SD, Schoeffel RE, and Finney M.** Evaluation of ultrasonically nebulized solutions for provocation testing in patients with asthma. *Thorax* 38: 284–291, 1983.
4. **Caterina MJ, Rosen TA, Tominaga M, Brake AJ, and Julius D.** A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* 398: 436–441, 1999.
5. **Cowley EA, Govindaraju K, Loyd DK, and Eidelman DH.** Airway surface fluid composition in the rat determined by capillary electrophoresis. *Am J Physiol Lung Cell Mol Physiol* 273: L895–L899, 1997.
6. **Cowley EA, Govindarajy K, Guilbault C, Radzioch D, and Eidelman DH.** Airway surface liquid composition in mice. *Am J Physiol Lung Cell Mol Physiol* 278: L1213–L1220, 2000.
7. **Docherty RJ, Yeats JC, and Piper AS.** Capsazepine block of voltage-activated calcium channels in adult rat dorsal root ganglion neurons in culture. *Br J Pharmacol* 121: 1461–1467, 1997.
8. **Elia C, Bucca C, Rolla G, Scappaticci E, and Cantino D.** A freeze-fracture study of human bronchial epithelium in normal, bronchitis and asthmatic subjects. *J Submicrosc Cytol Pathol* 20: 509–517, 1998.
9. **Emonds-Alt X, Vilain P, Goulaouic P, Prietto V, Van Broeck D, Advenier DC, Naline E, Neliat G, Le Fur G, and Breliere JC.** A potent and selective non-peptide antagonist of the neurokinin A (NK2) receptor. *Life Sci* 50: 101–106, 1992.
10. **Fernadez-Fernadez JM, Nobles M, Curid A, Vazquez E, and Valverde MA.** Maxi K channel mediates regulatory volume decrease response in a human bronchial epithelial cell line. *Am J Physiol Cell Physiol* 283: C1705–C1714, 2002.
11. **Finney MJ, Anderson SD, and Black JL.** The effect of non-isotonic solutions on human isolated airway smooth muscle. *Respir Physiol* 69: 277–286, 1987.
12. **Gao X, Wu L, and O'Neil RG.** Temperature-modulated diversity of TRPV4 channel gating: activation by physical stresses and phorbol ester derivatives through protein kinase C-dependent and -independent pathway. *J Biol Chem* 278: 27129–27137, 2003.
13. **Green KA, Small RC, and Foster RW.** The properties of voltage-operated Ca^{2+} channels in bovine isolated trachealis cells. *Pulm Pharmacol* 6: 49–62, 1993.
14. **Güler AD, Lee H, Lida T, Shimizu I, Tominaga M, and Caterina M.** Heat-evoked activation of the ion channel, TRPV4. *J Neurosci* 22: 6408–6414, 2002.
15. **Gunthorpe MJ, Benham CD, Randall AD, and Davis JB.** The diversity in the vanilloid (TRPV) receptor family of ion channels. *Trends Pharmacol Sci* 23: 183–191, 2002.

Downloaded from http://ajplung.physiology.org/ by 10.220.33.2 on October 13, 2017

16. **Hisada T, Kurachi Y, and Sugimoto T.** Properties of membrane currents in isolated smooth muscle cells from guinea-pig trachea. *Pflügers Arch* 416: 151–161, 1990.
17. **Jia Y, McLeod RL, Wang X, Parra LE, Egan RW, and Hey JA.** Anandamide induces cough in conscious guinea pigs through VR1 receptors. *Br J Pharmacol* 137: 831–836, 2002.
18. **Jia Y, Wang X, Aponte SI, Rivelli MA, Yang R, Rizzo CA, Corboz MR, Priestley T, and Hey JA.** Nociceptin/orphanin FQ inhibits capsaicin-induced guinea pig airway contraction through an inward-rectifier potassium channel. *Br J Pharmacol* 135: 764–770, 2002.
19. **Jia Y, Xu L, Turner DJ, and Martin JG.** Endogenous nitric oxide contributes to strain-related differences in airway responsiveness in rats. *J Appl Physiol* 80: 404–410, 1996.
20. **Jongejan RC, De Jongste JC, Raatgeep RC, Bonta IL, and Kerrebijn KF.** Effects of changes in osmolarity on isolated human airways. *J Appl Physiol* 68: 1568–1575, 1990.
21. **Joris E, Dab I, and Quinton PM.** Elemental composition of human airway surface fluid in healthy and diseased airways. *Am Rev Respir Dis* 148: 1633–1637, 1993.
22. **Kotlikoff MI.** Calcium currents in isolated canine airway smooth muscle cells. *Am J Physiol Cell Physiol* 254: C794–C801, 1988.
23. **Laitinen L, Heino M, Laitinen A, Kava T, and Haahtela T.** Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Respir Dis* 131: 599–606, 1985.
24. **Liedtke W, Choe Y, Marti-Renom MA, Bell MA, Denis CS, Sali A, Hudspeth AJ, Friedman JM, and Heller S.** Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell* 103: 525–535, 2000.
25. **Liedtke W and Friedman JM.** Abnormal osmotic regulation in trpv4$^{-/-}$ mice. *Proc Natl Acad Sci USA* 100: 13698–13703, 2003.
26. **Lou YP, Lee LY, Satoh H, and Lundberg JM.** Postjunctional inhibitory effect of the NK2 receptor antagonist, SR 48968, on sensory NANC bronchoconstriction in the guinea pig. *Br J Pharmacol* 109: 765–773, 1993.
27. **McLean S, Ganong A, Seymour PA, Snider RM, Desai MC, Rosen T, Bryce DK, Longo KP, Reynolds LS, Robinson G, Schmidt AW, Siok C, and Geym J.** Pharmacology of CP-99994, a nonpeptide antagonist of the tachykinin neurokinin-1 receptor. *J Pharmacol Exp Ther* 267: 472–479, 1993.
28. **Mizuno A, Matsumoto N, Imai M, and Suzuki M.** Impaired osmotic sensation in mice lacking TRPV4. *Am J Physiol Cell Physiol* 285: C96–C101, 2003.
29. **Nilius B, Prenen J, Wissenbach U, Bodding M, and Droogmans G.** Differential activation of the volume-regulated anion currents in HEK-293 cells. *Pflügers Arch* 443: 227–233, 2001.
30. **Nilius B, Vriens J, Prenen J, Droogmans G, and Voets T.** TRPV4 calcium entry channel: a paradigm for gating diversity. *Am J Physiol Cell Physiol* 286: C195–C205, 2004.
31. **Rodwell LT, Anderson SD, and Seale JP.** Inhaled clemastine, an H1 antihistamine, inhibits airway narrowing caused by aerosols of non-isotonic saline. *Eur Respir J* 4: 1126–1134, 1991.
32. **Rizzo C, Valentine AF, Egan RW, and Hey JA.** NK2-receptor mediated contraction in monkey, guinea-pig and human airway smooth muscle. *Neuropeptides* 33: 27–34, 1999.
33. **Schoeffel RE, Anderson SD, and Altounyan RECB.** Bronchial hyperreactivity in response to inhalation of ultrasonically nebulised solutions of distilled water and saline. *Br Med J* 283: 1285–1287, 1981.
34. **Strotmann R, Harteneck C, Nunnenmacher K, Schultz G, and Plant TD.** OTRPV4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. *Nat Cell Biol* 2: 695–702, 2002.
35. **Suzuki M, Mizuno A, Kodaira K, and Imai M.** Impaired pressure sensation in mice lacking TRPV4. *J Biol Chem* 278: 22664–22668, 2003.
36. **Voets T, Prenen J, Viens J, Watanabe H, Janssens A, Wissenbach U, Bodding M, Droogmans G, and Nillius B.** Molecular determinants of permeation through the cation channel TRPV4. *J Biol Chem* 277: 33704–33710, 2002.
37. **Vriens J, Watanabe H, Janssens A, Groogmans G, Voets T, and Nillius B.** Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. *Proc Natl Acad Sci USA* 101: 396–401, 2004.
38. **Watanabe H, Davis JB, Smart D, Jerman JC, Smith GD, Hayes P, Vriens J, Cairns W, Wissenbach U, Prenen J, Flockerzi V, Droogmans G, Benham CD, and Nilius B.** Activation of TRPV4 channels (hVRL-2/mTRP12) by phorbol derivatives. *J Biol Chem* 277: 13569–13577, 2002.
39. **Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, and Nillius B.** Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature* 424: 434–438, 2003.
40. **Watanabe H, Vriens J, Suh SH, Benham CD, Droogmans G, and Nilius B.** Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells. *J Biol Chem* 277: 47044–47051, 2002.
41. **Wissenbach U, Bodding M, Freichel M, and Flockerzi V.** Trp12, a novel Trp-related protein from kidney. *FEBS Lett* 485: 127–134, 2000.
42. **Yamada A, Sato O, Watanabe M, Walsh MP, Ogawa Y, and Imaizumi Y.** Inhibition of smooth muscle myosin-light-chain phosphatase by ruthenium red. *Biochem J* 349: 797–804, 2000.

Downloaded from http://ajplung.physiology.org/ by 10.220.33.2 on October 13, 2017