Functional TRPV4 channels are expressed in human airway smooth muscle cells


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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, confirmed by antinociceptive 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 constriction 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...
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 Accreditation and Accreditation of Laboratory Animal Care-accredited program.

Materials. Capsaicin, capsazinpe, carbobal, 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 Whitaker (Walkersville, MD) and maintained in smooth muscle cell growth media (SmGM-2, Bio Whitaker) at 37°C in humidified air containing 5% CO2. HBSMC were subcultured into black-welled, clear-based 96-well plates, and confluent cells from the 4th to 7th passages were used for intracellular Ca2+ 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’- CAGGCCCTCTATGACTCGAGGA, 3’-CAGTGATCCGCGTTGIGA; TRPV4: 5’-GAGGCAATCGGCAACAGA, 3’-GCCGCTTGTGCTCTC- ATCCGTC; TRPV2: 5’-GAGGAAATCGGCAACAGA, 3’-GCCGT- GTGTCCTCCTGTC; Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers (Clontech) were used as a positive control. The resulting PCR products for TRPV4 and TRPV2 were subcloned and sequenced for verification of RT-PCR amplification.

Measurements of intracellular Ca2+ concentration using the fluorometric imaging plate reader. Intracellular Ca2+ 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 Ca2+-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 osmol/l per liter). The plates were then placed into a FLIPR (Molecular Devices). Intracellular Ca2+ 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 mmHg) and isotonic buffer (310 mmHg) were elicited by direct addition of the hypotonic buffer or isotonic buffer, respectively. The responses were recorded for 10 minutes.

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 primers 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. 2). Using cationic primers 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 Ca2+ influx through the channel. The function of TRPV4 was characterized in primary cultured human airway smooth muscle cells. Intracellular Ca2+ level in response to hypotonicity in cultured cells was measured using the FLIPR technique. Hypotonic stimulation (90–70% of isotonic solutions) increased intracellular Ca2+ concentration in an osmolarity-dependent manner in cultured HBSMC (Fig. 2). The Ca2+ response to hypotonic buffer was observed only when Ca2+ was present in the extracellular buffer, indicating a Ca2+ influx through a membrane ion channel (Fig. 2). Capsaicin (10 μM), a TRPV1 agonist, had no effect on intracellular Ca2+ in the cell (data not shown).
TRPV4 is also activated by the phorbol derivative 4α-PDD (38). In the present study, 4α-PDD also induced Ca2+ influx in cultured HBSMC only when Ca2+ was present in the extracellular solution (Fig. 3A) but not in Ca2+-free conditions (Fig. 3B). When cells were pretreated with ruthenium red (1 μM, 10 min), a TRPV4 blocker (36, 38), the 4α-PDD-induced Ca2+ response was inhibited by 75%. On the contrary, the 4α-PDD-induced Ca2+ response was not inhibited by capsazepine, a TRPV1 channel inhibitor, or by verapamil, an L-type电压门控钙通道 (VGCC) inhibitor (Fig. 3B).

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. 4A, hypotonic buffer induced smooth muscle contraction in human bronchus in the presence of Ca2+. When Ca2+ was removed from the buffer, hypotonicity-induced airway smooth muscle contraction was significantly decreased (Fig. 4A, 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 Ca2+ (Fig. 4B). Removal of airway epithelium did not change the contraction induced by hypotonic stimulation in guinea pig trachealis (Fig. 4B, P = 0.34). Hypotonic stimulation-induced airway contraction was abolished in the Ca2+-free condition and was restored by adding Ca2+ to the buffer (Fig. 4C). On the other hand, carbachol-induced contraction is decreased, but not abolished, in the Ca2+-free condition (Fig. 4C).

Pharmacological regulation of the hypotonicity-induced airway contraction was tested next, using guinea pig trachealis. Nifedipine (1 μM), an L-type Ca2+ channel inhibitor, had no effect on hypotonicity-induced airway contraction (Fig. 5B), excluding the involvement of L-type Ca2+ channels. Similarly, capsazepine (1 μM), a TRPV1 channel antagonist, did not affect hypotonic stimulation-induced airway contraction (Fig. 5B). Capsazepine is also known to inhibit VGCC in rat dorsal root ganglion neurons with IC50 = 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
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 \mu M), an NK1 receptor antagonist (27), and SR-48968 (0.3 \mu M), 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. 5C). On the contrary, the same concentrations of CP-99994 and SR-48968 had no effect on hypotonicity-induced airway contraction (Fig. 5C), 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} \text{ 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
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) capsaicine, 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
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 tracheal tissue 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 tracheal tissue 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 mosmol 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.

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REFERENCES


