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Molecular and Functional Characterization of a Murine Calcium-activated Chloride Channel Expressed in Smooth Muscle*

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To identify the gene products responsible for the calcium-activated chloride current in smooth muscle, reverse transcription-PCR with degenerate primers was performed on mouse intestine and other organs. A new member of the CLCA gene family was identified, mCLCA4, that is expressed preferentially in organs containing a high percentage of smooth muscle cells, including intestine, stomach, uterus, bladder, and aorta. Reverse transcription-PCR using template RNA prepared from mouse bladder and stomach smooth muscle layers dissected free of mucosa yielded mCLCA4-specific bands. *In situ* hybridization with an mCLCA4-specific probe confirmed prominent expression in smooth muscle of major vessels of the heart but not cardiac muscle. High expression was also detected in the gastrointestinal tract, in bronchioles, and in aortic and lung endothelial cells. Transient expression of mCLCA4 in 293T cells resulted in the appearance of a prominent calcium-activated chloride current. Whole-cell currents activated by ionomycin or methacholine were anion-selective and showed minimal rectification or voltage-dependent gating. Similar to endogenous currents in smooth muscle cells, methacholine-induced currents were transient, and spontaneous transient inward currents were occasionally observed at resting membrane potentials. These results link calcium-activated chloride channels in smooth muscle with a gene family whose members have been implicated in cystic fibrosis, cancer, and asthma.

Calcium-activated chloride currents have been reported in a number of cell types including exocrine gland (1–3), smooth muscle (4–7), cardiac muscle (8, 9), epithelium (10–12), and endothelium (13). In vascular and non-vascular smooth muscle, calcium-activated chloride channels underlie one component of excitatory postsynaptic potentials (4–6, 14), and the local gating of Ca^{2+}-activated chloride channels by unitary Ca^{2+} release events results in spontaneous transient inward currents, termed STICs (15–17). Physiological evidence suggests that activation of $I_{Cl(Ca)}$[1] is an important component of rhythmic electrical activity (18, 19) and excitation/contraction coupling (20–22) in smooth muscle. Despite the prominent role of these channels in evoked and spontaneous electrical activity of smooth muscle, the molecular identity of the underlying channel remains unknown.

A family of calcium-activated chloride channels, termed CLCA, has recently been identified at the molecular level in many epithelial and endothelial cell types (23–30). CLCA family members have been implicated in pathological states such as asthma and cancer (30–33), although their function in normal cell physiology is not well established. Here we report the cloning and expression of a new member of the CLCA gene family in mouse. Unlike other CLCA genes, mCLCA4 is highly expressed in smooth muscle. Transient transfection of HEK293 cells with mCLCA4 results in the expression of calcium-activated currents with anion selectivity and kinetics similar to those observed in smooth muscle. These results suggest that mCLCA4 is the gene that encodes calcium-activated chloride channels in smooth muscle and broadens the physiological and pathological relevance of the CLCA gene family.

MATERIALS AND METHODS

Identification and Isolation of mCLCA4—RNA was prepared from mouse large intestine, kidney, and lung by grinding frozen organs with a mortar and pestle and extracting with Trizol (Invitrogen). 1 μg of RNA was reverse-transcribed (Superscript, Invitrogen) using random hexamers. cDNA was subjected to PCR (93 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s; 35 cycles) with degenerate primers based on Lu-ECAM-1 amino acids 36–45 (5′-ATTGCAATTAACCCCAGTGTGCCAGANGA-3′) and 165–174 (5′-GCRTAYTCRTCRAANAYNCCCCA-3′). PCR products were subjected to direct sequencing to rule out *Taq* PCR error. Distinct sequences were obtained from intestine *versus* kidney and lung (which were identical to mCLCA1) and were consistent across multiple RNA and cDNA preparations. The full open reading frame of mCLCA4 was obtained in two steps. Using oligo-dT-primed intestinal cDNA as a template and primers derived from the mCLCA1 sequence, a product was obtained comprising the start codon to base pair 2550. 3′ RACE was then employed to obtain the interval from base pair 2359 to the 3′ poly(A) tract. Primers derived from these sequences were used to amplify a 2.7-kb product containing the entire open reading frame. PCR error was minimized by using high fidelity Herculase DNA polymerase (Stratagene), by sequencing products directly before cloning, and by sequencing multiple pGEM-T clones. The final product was transferred as a *Sal*I/*Sst*II fragment to pIRES2 (*Xho*I/*Sst*II) for expression in HEK293T cells. One 3′ RACE product was obtained that contained an insertion at base pair 2663 (5′-TGGTCTGAGTACCCCCAGCACCCCT-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY00827.

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[1] The abbreviations used are: $I_{Cl(Ca)}$, calcium-activated chloride current; RACE, rapid amplification of cDNA ends; RT, reverse transcription.

CCTGGTCTGAGTACCCCCAGCACCCCTCCTGGTC-3′), resulting in a triple repeat of the amino acid sequence LSTPSTPPG beginning at position 880. The full-length clones all lacked the insertion.

Organ and Tissue Distribution of mCLCA4 by RT-PCR—Primer pairs specific for mCLCA4 were designed based on alignment of the four known mouse CLCA genes and corresponding to the interval base pairs 1123–1443, 5′-catcaatgacagctcctacctagc-3′ and 5′-atcaatcaggccattcacgtcttcc-3′. Primers were also designed to bracket at least one intron to rule out amplification of genomic DNA. Initially, a mouse multitissue cDNA array (RapidScan, Origene) was probed. To confirm and extend these results, the organs identified in the array and several others were isolated from a C57BL/6 mouse, and RNA was extracted and subjected to RT-PCR as before. Smooth muscle tissue was isolated from mouse bladder and stomach by dissecting away mucosal and serosal tissue layers under microscopic observation. 18 S ribosomal RNA was amplified as an internal control for RNA amount and reverse transcription using a primer:competitive oligomer ratio of 3:7 as described by Ambion. PCR was varied from 15 to 35 cycles to determine the range of linear amplification with added template. The profile at 25 cycles is shown. Total RNA from mouse aortic endothelial cells was a gift from Bernd Nilius (Leuven, Belgium). Lung microvascular endothelial cells were isolated from lung tumors as described (34). In some tissues a smaller product, identified by an *x* in Fig. 4, was sometimes obtained in addition to the expected band. This product was excised, inserted into pGEM-T (Promega), and determined by sequence analysis of three clones to be an artifact unrelated to mCLCA4. In addition, a Southern blot of the gel hybridized with mCLCA4 cDNA detected only the upper band.

In Situ Hybridization of Mouse Tissues—A subclone containing base pairs 2670 of the mCLCA4 open reading frame through the 3′-untranslated region was used for *in situ* hybridization analysis; this probe avoided regions with high similarity to mCLCA1 and mCLCA2. *In situ* hybridization studies were performed using a modification of procedures described by Wilkinson and Green (35). Mouse tissues were fixed overnight in freshly prepared ice-cold 4% paraformaldehyde in phosphate-buffered saline. The embryos were dehydrated through ethanol into xylene and embedded in paraffin using a Tissue-Tek V.I.P. automatic processor (Miles, Mishawaka, IN). Sections (5 μm) were adhered to commercially modified glass slides (Super Frost Plus, VWR, Rochester, NY), dewaxed in xylene, rehydrated through graded ethanols, and treated with proteinase K to enhance probe accessibility and with acetic anhydride to reduce nonspecific background. Single-stranded RNA probes were prepared by standard techniques with specific activities of 5×10^9 dpm/mg. Sections were hybridized at T_m − 15 °C, washed at high stringency (T_m − 7 °C) and treated with RNase A to further diminish nonspecific adherence of probe. Autoradiography with NBT-2 emulsion (Eastman Kodak Co.) was performed for 25 days. Slides were developed with D19 (Kodak), and the tissue was counterstained with hematoxylin.

Patch Clamp Recording—The expression of $I_{Cl(Ca)}$ in mCLCA4-transfected and control HEK293T cells was examined by whole-cell, patch clamp recording using classical and perforated patch clamp methods as described previously (36, 37). Briefly, HEK293T cells were transfected with green fluorescent protein-expressing pIRES2-mCLCA4 or vector alone (LipofectAMINE Plus, Invitrogen), trypsinized 24 h later, and seeded onto fibronectin-coated glass coverslips. After 18 h, cells were transferred to a temperature-controlled chamber maintained at 36 °C (Brook Industries) and superfused at 1 ml/min with extracellular solution (126 mM NaCl, 1.2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES, and 11 mM glucose; the pH was adjusted to 7.4 with NaOH. Anion shift experiments utilized the above extracellular solution in which 100 mM NaCl was replaced by an equimolar amount of sodium glutamate, shifting the theoretical chloride equilibrium potential to 24 mV (assuming zero glutamate permeability). Recording pipettes (resistance, 3–5 megaohms) were filled with 130 mM CsCl, 1.2 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 1 mM MgATP, and 0.075 mM EGTA; the pH was adjusted to 7.2 with CsOH. For perforated patch experiments, pipettes were dipped in pipette solution for 1–2 s then back-filled with pipette solution containing 200 mg/ml nystatin. After seal formation and establishment of the whole cell recording configuration, cells were voltage-clamped at −60 mV (Axopatch 200 B, Axon Instruments). Records were filtered at 500 Hz and sampled at 1 kHz. Current reversal potentials were measured either by step or ramp (−60 to 40 mV applied every 30 s) protocols. Cells were exposed to calcium-mobilizing agents (ionomycin or methacholine) by means of a puffer pipette connected to a controlled solenoid (Picospritzer, General Valve Corp.). A microscope equipped with an ultraviolet source was used to select green fluorescent protein-positive cells for recording.

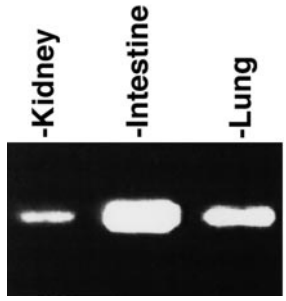

FIG. 1. **Identification of CLCA family members in mouse organs.** RNA was extracted from kidney, large intestine, and lung and subjected to RT-PCR with degenerate primers based on known CLCA amino acid sequences.

RESULTS

Identification and Cloning of a New CLCA Family Member—To identify the CLCA isoform present in mouse smooth muscle, RT-PCR was performed with degenerate primers on template RNA extracted from an organ rich in smooth muscle, the large intestine. Degenerate primers for RT-PCR were designed based on published CLCA sequences to amplify any CLCA cDNA including mCLCA1, mCLCA2, and mCLCA3. Thus, RNA extracted from established sites of mCLCA1 expression, lung and kidney, could serve as positive controls for amplification of CLCA mRNA. RT-PCR of intestinal RNA produced a much stronger band than either lung or kidney (Fig. 1). Direct sequencing of the amplification products revealed only the mCLCA1 sequence in lung and kidney but a new and distinct sequence in intestine, which we have designated mCLCA4. The electropherogram showed no sign of mCLCA3, previously reported in intestinal goblet cells, indicating that mCLCA4 is the predominant CLCA family member in large intestine (data not shown). The full 2.7-kb, 909-amino acid open reading frame of mCLCA4 was obtained using primers derived from the mCLCA1 sequence and 3′ RACE (Fig. 2). Comparison of the mCLCA4 amino acid and DNA sequences with those of the other mouse CLCA family members revealed an abrupt divergence at amino acid 873 resulting from a frameshift mutation (Fig. 2; GenBank™ accession number AY00827). mCLCA4 retains 79% identity with mCLCA1 and mCLCA2 but only 45% with mCLCA3. All of the general features found in other CLCA family members, such as the symmetrical cysteine cluster, processing sites, and glycosylation sites are conserved in mCLCA4 (Fig. 2). Construction of a phylogenetic tree of known CLCA proteins places mCLCA4 on Branch A, whose members all bear the sequence RARSPT (corresponding to amino acids 592–597 of mCLCA4), containing two adjacent sites for phosphorylation by calcium/calmodulin kinase II and protein kinase C as well as a site for protein kinase A (Fig. 3). Members on branches B and C lack this sequence (note that the genetic nomenclature is based on order of discovery; thus, mCLCA4 is not the ortholog of hCLCA4).

Tissue Distribution of mCLCA4 Expression—In preliminary experiments, to identify tissues in which mCLCA4 was expressed, a multi-organ mouse cDNA array (RapidScan, Origene) was probed by RT-PCR with primers that recognized mCLCA4 but not other mouse CLCA family members. Expression was detected in the gastrointestinal tract, uterus, and heart (data not shown). To confirm and extend these results, these and other organs were dissected and analyzed from a C57BL/6 mouse. Expression was highest in large and small intestine and was also significant in stomach and esophagus but just detectable in salivary gland (Fig. 4*A*; lower band *x* is an artifact). Outside the gastrointestinal tract, strong expression was detected in uterus and lung, and lower expression was

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mCLCA4 MVPGLQVLLFLTLHLLQNT-ESSMVHLNSNGYEGVVIAINPSVPEDERLIPSIKEMVTQASTYLFEATERRFYFRNVSILVPITWKSKTE 89
mCLCA1 ....................-.....................................................SQG.V....I.....M.....P. 89
mCLCA2 ....................-.....................................................SQG.V....I.....M.....S. 89
mCLCA3 .ESLKSPVFL.I....EGVLSE.LIQ..N.....I....DHD.....A..QH..D......P......GK....K..A..I.ES..A.P. 90

mCLCA4 YLTPKQESYDQADVIVADPHLQHGDDPYTLQYGQCGDRGQYIHFTPNFLLTDNLGIYGPRGRVFVHEWAHLRWGVFDEYNMDRPFYMSRK 179
mCLCA1 ..M..R....K.....................................................R.....................V.Q..... 179
mCLCA2 ..M..R....K.....................................................R.....................V.....I... 179
mCLCA3 .TR..L.TFKN...L.STTSPLGN.E...EHI.A..EK.IR..L..D..AGKK.TQ...QD.T.......F.....N...N.EK..L.-. 179

mCLCA4 NTVEATRCSTDITGTSVVRECQGGSCVSR-RCRRDAKTGMQEAKCTFIPNKSQTARGSIMFMQSLDSVVEFCTEKTHNVEAPNLQNKMCN 268
mCLCA1 ..I.......R....N..HN.ER.N..T.-A....S..RLY.P......D.I...GA......N.N.........N..A.......... 268
mCLCA2 ..I......AS...KK..H...R....T.-A....S..RLY.P......D.I...GA......N.N.........NN..A.......... 268
mCLCA3 GKPQ.V...AA...KNQ..R......ITNGK.VI.RV..LYKDN.V.V.DPH.NEKA....N.NIN.........N..Q....D..QR.. 269

mCLCA4 LRSTWDVIKASADFQNASPMTGTEAPPLPTFSLLKSRQRVVCLVLDKSGSMRLGSPITRLTLMNQAAELYLIQIIEKESLVGLVTFDSTA 358
mCLCA1 R........T.......P..R......P.........R.............---DKED..IR.........T..V....M........A. 355
mCLCA2 R..............SP..R......P.........R.............---DKED..IR.........T..V....M........A. 355
mCLCA3 .....E..QE.E..KQTT..--.AQ..A......QIG..I...........L---NDD..NR....SR.F.L.TV.QG.W..M.....A. 354

mCLCA4 TIQTNLIRIINDSSYLAISTKLPQYPNGGTSICNGLKKGFEAITSSDQSTSGSEIVLLTDGEDNRISSCFQEVKHSGAIIHTIALGPSAA 448
mCLCA1 H..NY..K.TSS.D.QK.TAN...QAS......H..QA..Q.......................G.R...EA.SR............... 445
mCLCA2 H..NY..K.TSS.D.QK.TAN...QAT......H..QA..Q.......................G.....EA.SR............... 445
mCLCA3 YV.SE.KQLNSGADRDLLIKH..TVSA......S..RTA.-TVIKKKYP.D.............T.....DL..Q.......V....A.. 443

mCLCA4 RELETLSDMTGGLRFYAKEDV--NGLIDAFSGISSKSGSISQQALQLESKAFNVGAGAWINSTVPVDSTVGNDTFFVITWTVRKPEIILQ 536
mCLCA1 .................NK.L--.S......R...T...V............D.R......G...L..............M.K....... 533
mCLCA2 .................NKH.--SS......R...T...V..............R.............................Q....... 533
mCLCA3 K...Q..K.....QT.SSDQ.QN...V...AAL..GNAA.A.HSI....RGV.LQNNQ.M.GS.I...S..K..L.L....THP.T.FIW 533

mCLCA4 DPKGKNYTTSDFQEDKLNIFSVRLRIPGIAETGTWTYSLLNKGATSQLLTVTVTTRARSPTTLPVIATAHMSQSTAQYPSRMIVYARVSQ 626
mCLCA1 .....K.......D.....R.A..Q...T.........I--T.TK...I.M..........ME.LL........................ 621
mCLCA2 .....K.I.....D.E...R.A..Q...T.........I--T.TK...I.M..........ME.LL...................V.... 621
mCLCA3 ..S.VE--QNG.IL.T-TTKVAY.QV..T.KV.F.K..I---Q.S..T..L...S..A.A.LP.ITV.PVVNKN.GKF..PVT...SIR. 617

mCLCA4 GFLPVLGANVTAVIEAESGNQVTLELWDNGAGADTLKNDGIYSRYFTDFHGNGRYSLKVNAQARKNMAKL-NLKQKNKSLYIPGYVENDQ 715
mCLCA1 ............L....H.H..............V......T.....Y..........RV..QR.KTR.-S.R..............GK 710
mCLCA2 ............I....H.H..............V......T.....Y..........RV.....K.R.-S.R..............GK 710
mCLCA3 .AS.I.R.S...L..SVN.KT.....L.......AT....V...F..A.DA.....V.IW.LGGVTSDRQRAAPP..RAM..D.WI.DGE 707

mCLCA4 IVLNPPRPEIPE-ATEATVEDFSRLTSGGSFTVSGAPPDGDHARVFPPSKVTDLEAEFIGDH-IHLTWTAPGKVLDKGRAYRYVIRMSGH 803
mCLCA1 ........DVQ.E.I.......N.V.....................................Y-.............N...H..I....Q. 799
mCLCA2 ........DVQ.E.I.......N.V.....................................Y-.Q.............H..I..V.Q. 799
mCLCA3 VRM......TS--YVQDKQLC...TS.....VATNV.AAAPIPDL...CQI...K.SIQ.QNLVN.......DDY.H...SN.I....TS 795

mCLCA4 SLALQEDFSNSTLVNTSSVMPKEAGSKETFKFKPETFKIENGTQVYIAIQADNEARLSSEVSNIAQAVKFIPPQVYLTPSTPPGLGTKVS 893
mCLCA1 P.D.....N.A....A..LI.....................A..I.L..........S.T...........LTSLE-----DSISA..DDI. 884
mCLCA2 P.G.....N.A....A..LI.....................A.D..L......Y...G.T...........TSLE-----DSISA..ADI. 884
mCLCA3 IVD.RDH.NT.LQ...TGLI....S.E.I.E.ELGGNTFG...DIF.....VDKSN.K..I....RVSV...A.EPPI.EDSTPPCPDI. 885

mCLCA4 VPSLT--VFVLVATL--F--------IF                                                              909
mCLCA1 AI.M.--IWG.TVIFNSI--------LN                                                              902
mCLCA2 AI.M.--.WG.AVIFNSI--------LN                                                              902
mCLCA3 IN.TIPGIH..KIMWKWLGEMQVTLGLH                                                              913
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FIG. 2. **Comparison of mCLCA4 amino acid sequence with those of other mouse CLCA family members.** Glycosylation sites are indicated by *stars*; the cysteine cluster is indicated by *underlines*, the phosphorylation hotspot is indicated by a *double underline* with *closed circles* indicating the phosphorylated residues, and the processing site by an *arrowhead*. Alignment was performed using the Megalign program, Clustal method, version 4.05 of the DNAStar package from Lasergene.

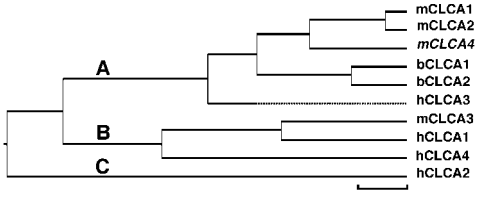

FIG. 3. **Dendrogram showing the relation of mCLCA4 (*italics*) to the other known family members.** Complete amino acid sequences were used to derive the tree using DNAStar software. The *bar* represents 10% sequence divergence. Note that nomenclature indicates order of discovery rather than orthology.

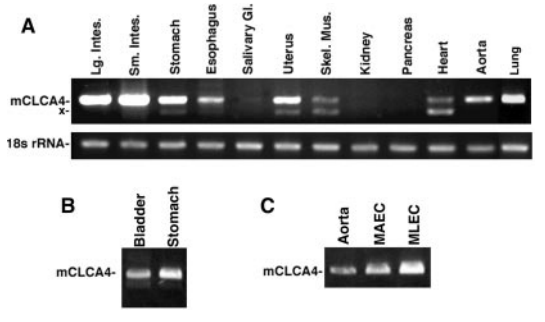

FIG. 4. **Tissue specificity of mCLCA4 expression determined by RT-PCR.** *A*, whole organs. *Lower band x* was sequenced and found to be an unrelated artifact. *B*, isolated smooth muscle from bladder and stomach. *C*, isolated endothelial cells. *MAEC*, mouse aortic endothelial cells. *MLEC*, mouse lung microvascular endothelial cells.

detected in aorta, heart, and skeletal muscle, whereas none was detectable in kidney or pancreas (Fig. 4*A*). To determine whether mCLCA4 expression in mixed organs was associated

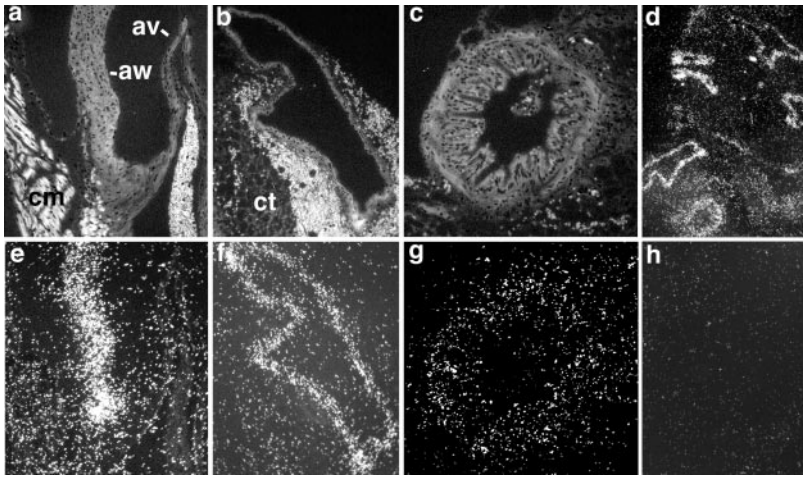

FIG. 5. **Expression of mCLCA4 in vascular tissues determined by *in situ* hybridization of tissue sections.** A longitudinal section of the heart was hybridized with a ^{33}P-labeled mCLCA4 probe, exposed to photo-emulsion, and examined by UV (ultraviolet autofluorescence, *a–c*) or dark-field optics (*d–h*). Significant hybridization was observed in aorta (*a* and *e*), right pulmonary vein (*b* and *f*), coronary artery (*c* and *g*), and all other vessels in the section (*d*) with the antisense RNA probe, whereas the sense control produced no hybridization above background (*h*). Specimens were photographed using a 10× (*a* and *e*), 20× (*b*, *c*, *f*, and *g*) or 5× (*d* and *h*) objective. *aw*, aortic wall; *av*, aortic valve; *cm*, cardiac muscle; *ct*, connective tissue.

with expression in smooth muscle, two mouse organs were chosen in which the smooth muscle layer could be dissected free of overlying tissue layers. The tunica muscularis was dissected from the mouse bladder and stomach for RNA preparation and RT-PCR. As shown in Fig. 4*B*, isolated smooth muscle from both tissues was strongly positive for mCLCA4 expression, with stomach more prominent.

Cellular Expression of mCLCA4—To determine the sites of expression at higher resolution, we probed tissue sections by *in situ* hybridization with an mCLCA4-specific ^{33}P-labeled cRNA probe derived from the 3′-untranslated region. The best evidence for expression in smooth muscle was observed in vessels associated with the heart. A very strong signal was observed in the walls of the aorta with a much lower signal in adjoining cardiac muscle and little signal above background in the endothelium of the aortic valve (Fig. 5, *a* and *e*). The pulmonary vein (Fig. 5, *b* and *f*) and its branches were also intensely labeled, whereas the coronary artery was less so (Fig. 5, *c* and *g*). Connective and adipose tissues were consistently negative. The atrioventricular bundle, consisting of muscle cells modified to function as nerve fibers, was also positive (not shown). In lung, the signal was concentrated around bronchioles and blood vessels (Fig. 6, *a–d*). Surprisingly, in the gastrointestinal tract mCLCA4 expression was more strongly associated with the mucosa than with underlying smooth muscle (Fig. 6, *e–h*). In small intestine for example, label was concentrated in villi, whereas smooth muscle layers were more weakly labeled. In contrast to mCLCA1 and mCLCA3, mCLCA4 signal was not confined to deep crypt or goblet cells.

The aortic wall in mouse consists mostly of smooth muscle with only a single layer of endothelial cells lining the vessel interior. Because endothelial cells are difficult to detect by *in situ* hybridization, we obtained endothelial cells that had been isolated from aorta or lung microvasculature and analyzed them by RT-PCR. Endothelial cells from either source had high levels of mCLCA4 expression (Fig. 4*C*).

mCLCA4 Mediates a Calcium-activated Chloride Current—To determine whether mCLCA4 encodes a functional chloride channel, the cDNA was inserted into a vector bearing a green fluorescent protein marker and transfected into HEK293T cells, and whole cell currents of fluorescent cells were measured. Recordings were made in extracellular solution containing Cs^+ substituted for Na^+ to block any calcium-activated potassium currents. Under these conditions, mCLCA4-transfected HEK cells, but not vector-transfected cells, exhibited a prominent inward current when exposed to ionomycin (Fig. 7*A*). 16 of 23 cells exposed to ionomycin displayed prominent inward currents, whereas no current was

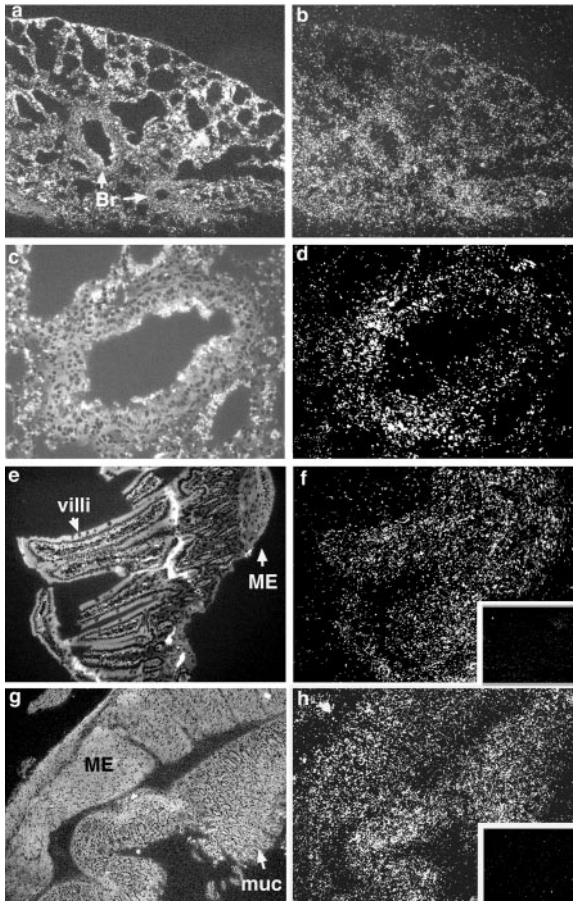

FIG. 6. **Expression of mCLCA4 in lung and gastrointestinal tract.** Sections of lung (*a–d*), small intestine (*e* and *f*), and stomach (*g* and *h*) were analyzed as in Fig. 5. *Left column*, UV; *right column*, dark-field microscopy. *Insets*, hybridization with sense probe control. Magnification of objective: *a* and *b*, 10×; *c* and *d*, 40×; *e–h*, 20×. *Br*, bronchiole; *ME*, muscularis externa; *Muc*, mucosa.

observed in 6 cells transfected with vector alone. The evoked currents were transient in nature even when evoked after exposure to ionomycin, which results in a sustained increase in $[Ca^{2+}]_i$; inactivation of the smooth muscle calcium-activated chloride channel that is independent of Ca^{2+} has been reported (38). Methacholine was also used to stimulate calcium release via endogenous muscarinic receptors. As shown in Fig. 7*B*, methacholine activated a transient current of similar time course and smaller magnitude than ionomycin (Fig. 7*B*). A

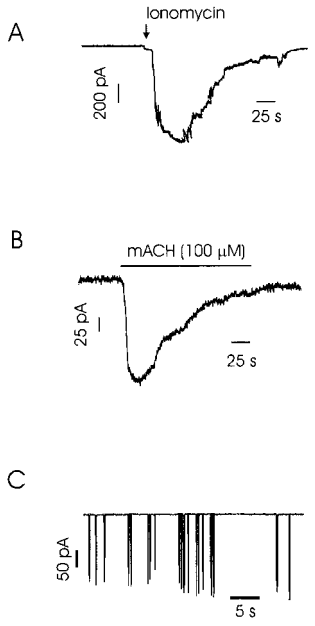

FIG. 7. **Transfection of mCLCA4 results in expression of a calcium-activated inward current.** *A*, exposure of HEK293T cells transfected with mCLCA4 to ionomycin (10 μM) activates a transient inward current. No currents were observed in non-transfected cells. *B*, exposure of these cells to methacholine (*mACH*) evoked a similar transient current with faster activation kinetics, consistent with a release of intracellular calcium stores. No inward currents were observed in vector-transfected control cells. *C*, in some mCLCA4-transfected cells spontaneous, transient inward currents were observed. These spontaneous transient inward currents were not observed in all cells but were never observed in non-transfected cells.

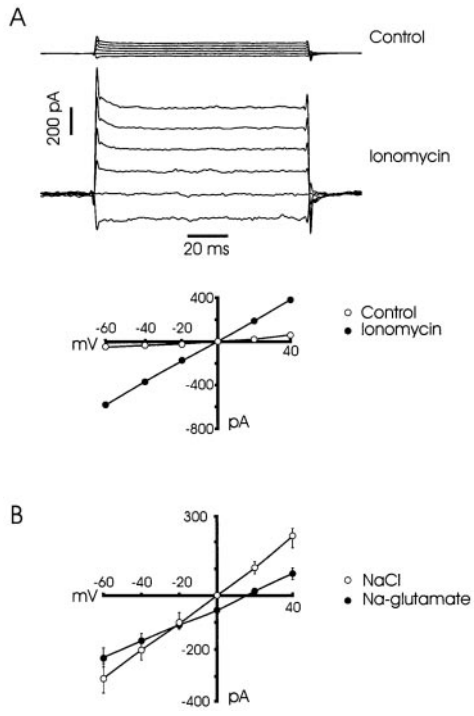

FIG. 8. **The mCLCA4 current is a calcium-activated chloride current.** *A*, during exposure of mCLCA4-transfected HEK293T cells to ionomycin, step depolarization was imposed to obtain the current/voltage relationship. The magnitudes of the currents at the end of the step are plotted below. Note that the current reversal potential is ~0 mV in symmetrical chloride solution. *B*, the shift in current reversal potential is shown for a cell recorded in symmetrical chloride solution and in solution in which chloride was replaced by glutamate in the extracellular solution. As shown, the current reversal potential was shifted to close to the theoretical chloride equilibrium potential (24 mV).

second prominent feature of expression of mCLCA4 was the appearance of spontaneous transient inward currents (STICs) similar to those observed in smooth muscle cells from several tissues (17, 39). Spontaneous currents were observed in 10 of 29 cells transfected with mCLCA4 (Fig. 7*C*), whereas in 21 cells transfected with a green fluorescent protein plasmid lacking mCLCA4 no spontaneous methacholine-activated chloride currents were observed.

To obtain the current-voltage relationship of the calcium-activated inward current, a step depolarization experiment was performed. As shown in Fig. 8*A*, the I-V relationship was linear over the voltage range from −60 to 40 mV, indicating little voltage dependence of gating or intrinsic rectification of mCLCA4 over this voltage range, similar to findings for mCLCA1 (25) and consistent with the electrophysiological behavior of these channels in smooth muscle (20). The current was shown to be chloride-selective by substitution of extracellular Cl^- ions with glutamate ions (Fig. 8*B*). In these experiments, the current was activated by exposure of cells to ionomycin in the presence of normal extracellular solution or the same solution in which 100 mM NaCl was replaced by 100 mM sodium glutamate; glutamate ions have a permeability ratio in calcium-activated chloride channels of approximately 0.05 relative to Cl^- ions (1, 40). In the presence of glutamate ions substituted for chloride ions, the reversal potential of the calcium-activated inward current shifted from 0 to 18.2 ± 3.6 mV (n = 6; Fig. 8*B*), which was quite close to the theoretical E_{Cl} (24 mV, assuming zero glutamate permeability) and confirmed the anion selectivity of the channel.

DISCUSSION

The CLCA gene family comprises an expanding group of calcium-activated chloride channels. To date, the expression of known CLCA genes has been largely restricted to epithelial and endothelial tissue, from which they were first cloned (23, 24). Despite early reports of prominent calcium-activated chloride currents in *Xenopus* oocytes (41), neurons (42, 43), secretory gland (1), and smooth muscle cells (4), in large measure the genes responsible for these currents have not been clearly identified, and the extent to which they represent variants of the described CLCA or ClC (44) gene families has been uncertain. The identification of a CLCA gene with prominent expression patterns in smooth muscle significantly expands the potential importance of this gene family.

In general, calcium-activated chloride channels in various mammalian tissues are similar in single-channel conductance, anion selectivity, and drug sensitivity (for review, see Ref. 45). These and other shared properties led to the proposal that all calcium-activated chloride channels in mammalian tissues may be mediated by a family of closely related proteins (20). Although the CLCA family is deeply divergent at the amino acid level, all share several features. The CLCA precursor is about 900 amino acids long and contains two proteolytic cleavage sites. The first cleavage removes the amino-terminal signal sequence, resulting in a long extracellular amino-terminal tail. The second cleavage event removes the carboxyl-terminal ~200 amino acids, yielding products of 90 and 30–40 kDa, both closely associated with the plasma membrane (24–27). Another signatory feature is a symmetrical cluster of cysteines, $CX_{12}CX_4CX_4CX_{12}C$, around amino acid 200. The structure and stoichiometry of the channel remain to be established.

We report the cloning and functional expression of a novel member of the CLCA family, termed mCLCA4. The gene product is strongly related to mCLCA1 and mCLCA2 and shares the potential phosphorylation sites for calcium/calmodulin kinase II, consistent with regulation by intracellular calcium.

RT-PCR and *in situ* hybridization analysis indicates substantial expression of mCLCA4 in smooth muscle and certain epithelial and endothelial tissues. Expression in smooth muscle was never observed for mCLCA1, mCLCA2, or mCLCA3 (46, 47, 31) and suggests that mCLCA4 may be associated with chloride currents recorded in this tissue. Expression studies with mCLCA4 were consistent with this notion, and transfection of HEK293T cells with mCLCA4 resulted in the expression of a prominent calcium-activated chloride current. The current-voltage relationship measured in voltage steps during exposure to ionomycin was consistent with a very weakly voltage-dependent channel, similar to measurements of calcium-activated chloride currents in smooth muscle (20) and other tissues (1, 41). Similarly, despite sustained elevations of $[Ca^{2+}]_i$ obtained with ionomycin, the activated current decayed with kinetics generally similar to that observed in smooth muscle (38). The appearance of spontaneous, transient calcium-activated currents, a prominent feature of smooth muscle chloride currents (17), also suggests a marked functional similarity between mCLCA4 and the smooth muscle channel.

In summary, we report the sequence and functional expression of a novel calcium-activated chloride channel with distinct tissue expression. Although the definitive determination of the extent to which this channel underlies specific postsynaptic currents in smooth muscle or secretory cells will require careful comparison of biophysical properties of heterologously expressed mCLCA4 with currents recorded in native cells and gene targeting experiments, our data identify mCLCA4 as a likely candidate for calcium-activated chloride currents in smooth muscle.

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Note Added in Proof—The dependence of STICs upon unitary calcium-release events was established by Walsh and co-workers (Zhu Ge, R., Sims, S. M., Taft, R. A., Fogarty, K. E., and Walsh, J. V. (1998) *J. Physiol.* (*Lond.*) **513,** 711–718).

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