Cloning of an Epithelial Chloride Channel from Bovine Trachea*

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We have isolated and cloned a novel epithelial Cl⁻ channel protein from a bovine tracheal cDNA expression library using an antibody probe. The antibody (αp38) was raised against a 38-kDa component of a homopolymeric protein that behaves as a Ca²⁺/calmodulin kinase II-, DIDS-, and dithiothreitol (DTT)-sensitive, anion-selective channel when incorporated into planar lipid bilayers. The full-length cDNA is 3001 base pairs long and codes for a 903-amino acid protein. The clone does not show any significant homology to any other previously reported Cl⁻ channel sequence. Northern analysis of bovine tracheal mRNA with a cDNA probe corresponding to the cloned sequence revealed a band at 3.1 kilobases, suggesting that close to the full-length sequence has been cloned. The full-length open reading frame (2712 base pairs) has been expressed in Xenopus oocytes and in mammalian COS-7 cells. In oocytes, expression of the clone was associated with the appearance of a novel DIDS-, and DTT-sensitive, anion-selective conductance that was outwardly rectified and exhibited a reversal potential close to 0 mV. Whole-cell patch clamp studies in COS-7 cells transfected with the clone identified an ionomycin-, and DTT-sensitive chloride conductance that was not apparent in mock-transfected or control cells. In vitro translation studies have shown that the primary transcript codes for a protein migrating at 140 kDa under reduced conditions, significantly larger than the polypeptide recognized by αp38. We therefore suggest that either the 140-kDa translated product is a prepro-form of the 38-kDa subunit of the previously identified bovine tracheal anion channel and that the primary transcript is post-translationally cleaved to yield the final product, or that the cloned channel and the previously identified bovine tracheal anion channel protein share an epitope that is recognized by the αp38 antibody.

Recent experimental evidence indicates that epithelia such as the mammalian trachea, kidney, and intestine contain different types of Cl⁻ channel including those sensitive to cAMP, Ca²⁺, voltage, and voltage (1). Chloride channels may be located on both basolateral and apical membranes, where they participate in NaCl transport in both absorptive and secretory cells (2). However, studies of epithelial Cl⁻ channels at the biochemical and molecular level have been severely limited because of a lack of appropriate pharmacological and molecular probes.

Until very recently, the primary amino acid sequence for many of these anion channels was unknown. The first epithelial Cl⁻ channel to be cloned was the (CFTR) protein (3). CFTR acts as a small conductance, linear, cAMP/protein kinase A-sensitive anion channel that is insensitive to inhibition by DIDS (1, 4), and exhibits an anion selectivity of Br⁻ > Cl⁻ > I⁻. Subsequently, Jentsch and co-workers (5) cloned and expressed a voltage-dependent Cl⁻ channel (CIC-2) from rat heart and brain. This clone encodes a protein with a predicted M₀ of 99,000 and is 50% homologous to both the Torpedo electroplax Cl⁻ channel (CIC-0) and rat muscle Cl⁻ channel (CIC-1; Refs. 6 and 7). The message for this CIC-2 channel was found by Northern analysis in a number of epithelial cell types, including the pancreas, kidney, intestine, and lung, and in several cell lines, such as T₈₄ cells, Chinese hamster ovary cells, and COS cells, and in the CFPAC-1 cell line. Uchida et al. (8) cloned a rat kidney Cl⁻ channel (CIC-K1), using degenerate oligonucleotide primers constructed from conserved regions of CIC-0, -1, and -2 in a PCR-based cloning strategy. This channel was primarily expressed in renal thick ascending limb. The currents expressed in oocytes were DIDS-sensitive, had a selectivity of Br⁻ > Cl⁻ > I⁻, and were postulated to be regulated by osmotic stress in vivo. A protein kinase A-sensitive gastric chloride channel, highly homologous to CIC-2, has also been isolated from the rabbit stomach (9), the selectivity of which was reported to be I⁻ > Cl⁻ > NO₃⁻ (10). Subsequently, Jentsch and co-workers (5) cloned and expressed a protein termed pClᵣ, the expression of which was associated with the appearance of an outwardly rectifying, Ca²⁺-insensitive, DIDS- and NPPB-blockable Cl⁻ channel with a predicted M₀ of 26,000. Unlike CFTR, nucleotides can also block this channel. Landry et al. (11) cloned a 64-kDa protein that functions as a Cl⁻ channel in intracellular organelles.

Recent experiments using CFTR “knockout” mice have indicated that a Ca²⁺-mediated Cl⁻ secretory pathway is up-regulated in the nasal mucosa of these animals and that Cl⁻ secretion is therefore a process molecularly distinct from activation of the CFTR pathway in these dfr/ dfr mice (12, 13). It has been shown that this Ca²⁺-dependent Cl⁻ channel can compensate for the lack of CFTR in the CFTR knockout mouse and

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§The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, 4,4-diisothiocyanostilbene-2,2'-disulfonic acid; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; bp, base pair(s); DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino) benzonic acid; Ca-CC, calcium-activated chloride channel.
thus accounts for the absence of significant airway pathology in this cystic fibrosis mouse model. Transduction of normal CFTR in monolayers of human cystic fibrosis nasal epithelia using adenoviral vectors suppressed Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} secretion, indicating that functional expression of the calcium-mediated pathway is inversely dependent on the expression of functional CFTR (14). Thus, the Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} secretory pathway is of functional importance in human cystic fibrosis and may prove amenable to pharmacological manipulation and amelioration of the airway disease.

Our laboratory has purified and functionally reconstituted a Cl\textsuperscript{-} channel protein from bovine tracheal epithelium using anion and cation exchange chromatography followed by immunopurification (15, 16). We have shown that this protein is phosphorylated by Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II, and acts as a Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II-activated anion channel when reconstituted into planar lipid bilayers (17). However, when incorporated into planar lipid bilayers, this channel cannot be activated through phosphorylation by protein kinase A. The native form of this protein is 140 kDa and it forms anion-selective, 25-30 picosiemens channels in 150 mM NaCl (16) with an anion selectivity of I\textsuperscript{-} > NO\textsubscript{3}\textsuperscript{-} > Br\textsuperscript{-} > Cl\textsuperscript{-}. The channel is inactivated by disulfide reduction procedures, and the reduced form of the channel, which appears as a 36-38-kDa protein, is not associated with channel activity in planar lipid bilayers. Of the other Cl\textsuperscript{-} channels purified or cloned to date, none are Ca\textsuperscript{2+}-dependent, and all exhibit different anion discrimination profiles. We here report the cloning of a novel protein following screening of a bovine tracheal cDNA expression library with polyclonal antibodies generated from the reduced 38-kDa tracheal protein.

**EXPERIMENTAL PROCEDURES**

**Materials**

Molecular reagents were obtained from Promega (Madison, WI), Novagen (Madison, WI), New England Biolabs (Beverly, MA), U.S. Biochemical Corp., Perkin-Elmer, Invitrogen (San Diego, CA), DuPont NEN, Life Technologies, Inc., or Stratagene (La Jolla, CA), as indicated. Female Xenopus laevis were obtained from Xenopus I (Ann Arbor, MI). Radioisotopes were obtained from either DuPont NEN or ICN (Costa Mesa, CA). Forskolin and ionomycin were obtained from Calbiochem. 5-Bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium were purchased from Boehringer Mannheim (Mannheim, Germany). [\textsuperscript{125}I]iodo[\textsuperscript{35}S]sulfate and nitro blue tetrazolium were purchased from Avanti Polar Lipids (Birmingham, AL). The putative Cl\textsuperscript{-} channel protein cDNA was cloned into pBluescript using R408 helper phage. Double-stranded plasmid DNA was sequenced using the Sequenase kit (US Biological, Wakefield, MA) and the sequence was determined with the MacVector PLUS software package (Advanced Genetics, Inc., Cambridge, MA). The sequence was deposited in GenBank under the accession number X53107.

**Methods**

**Isolation of cDNA Clones—**Bovine tracheal epithelium was scraped from fresh trachea obtained from a local slaughterhouse and immediately added to the lysis buffer of the Fast Track mRNA isolation kit (Invitrogen). Poly(A\textsuperscript{+}) mRNA isolation was then performed according to the manufacturer’s instructions. A cDNA library was constructed in the Unizap l vector (Stratagene) using 5 \mu g of tracheal poly(A\textsuperscript{+}) mRNA, according to the manufacturer’s instructions. A polyclonal antibody (IgG, raised against a tracheal Cl\textsuperscript{-} channel (18), was used to screen 4 \times 10\textsuperscript{9} plaques. In the initial screening, nine positive plaques were identified and purified by three further rounds of immunoscreening. All positives were excised into pBluescript using R408 helper phage. Double-stranded DNA was subjected to alkaline denaturation and sequenced by a modified Sanger dideoxynucleotide protocol using Sequenase 2.0 (US Biological), and [\textsuperscript{35}S]ATP (DuPont NEN). All computer analyses were performed using the Genetics Computer Group sequence analysis software package (Center for AIDS Research, University of Alabama at Birmingham). Northern and Southern Blot Analysis—Northern blot analysis was carried out with bovine poly(A\textsuperscript{+}) mRNA isolated from the trachea (Invitrogen Fast Track kit). RNA (2 \mu g) was electrophoresed through a denaturing 1% agarose/formaldehyde gel and transferred to GeneScreen Plus (DuPont NEN). The membrane was prehybridized in 1X SDS, 1 mM NaCl, 10% dextran sulfate at 60°C and hybridized with a [\textsuperscript{32}P]CTP probe at 2 \times 10\textsuperscript{6} cpm/ml. Following overnight hybridization, the blot was washed under high stringency prior to autoradiography. Southern analysis was carried out using a Zoo blot (Clontech), cut with EcoRI, and prehybridized in 5 X SSPE (150 mM NaCl, 10 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 7.4, 1 mM Na\textsubscript{2}EDTA), 10 \times Denhardt’s solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), and 100 mg/ml of sheared salmon sperm DNA. A XhoI restriction-fragment of the Ca-CC DNA clone, encompassing bases 1785-2985, was labeled with [\textsuperscript{32}P]CTP (DuPont NEN), using random primers and hybridized to the blot (1 \times 10\textsuperscript{6} cpm/ml) overnight at 65°C. The blot was washed under reduced stringency (2 X SSC, 0.05% SDS) at 60°C prior to drying and autoradiography.

**Analysis—**Bovine tissue RNAs from trachea, lung, brain, renal papilla, and liver were isolated as described (19). RNA from control Xenopus oocytes was isolated by homogenization in buffer containing 1X SDS as described (20). Reverse transcription of 1 \mu g of total isolated RNA was performed using avian myeloblastosis virus reverse transcriptase (Promega) for 60 min at 42°C using d(giol)dT\textsubscript{12} (Promega) to prime the reaction. Approximately 200 ng of the reverse transcribed cDNA was amplified by PCR for 30 cycles using 2 units of Taq polymerase (Promega) reaction, under the following conditions: denaturation, 94°C, 45 s; annealing, 58°C, 45 s; extension, 72°C, 2 min. Minors were designed corresponding to a 24-bp region bracketing the initial ATG codon (5'-ATAATGCGGCTGCTTGACTGTC-3') and to a 21-nucleotide region initiating at 704-bp downstream (5'-ACTCTTTGCTGTGTTGGATT-3'). In each case, primer design was based on a 150-bp product from bovine \textalpha-actin were used in parallel reactions as controls. The sense and antisense \textalpha-actin primers were 5'-CAATTGGGCCCATCTATAGGG-3' and 5'-GCTTCTTTTGTGTCGCCG-3', respectively. In the case of PCR reactions using transcribed Xenopus RNA, human \beta-actin primers (Stratagene) that amplified a 650-bp product were used for control PCR reactions. PCR products were electrophoresed through 2% agarose (Nu-Sieve, FMC)/Tris-borate-EDTA gels and stained with ethidium bromide.

Subcloning into Xenopus and pMT3 Expression Vectors—A modified version of pGEM11zf\textsuperscript{+} containing the 3'- and 5'-untranslated regions of the Xenopus \beta-globin gene with BglII restriction sites 3' and 5' to the insert, was a kind gift of Dr. Doug Melton (Harvard University, Cambridge MA). The putative Cl\textsuperscript{-} channel insert subcloned into the vector was obtained by PCR of the 2712-bp open reading frame using Vent thermosable DNA polymerase (New England Biolabs), under the following conditions: denaturation, 94°C, 1 min; annealing, 52°C, 1 min; extension, 72°C, 3 min; 30 cycles; and purification by gel electrophoresis. Two adaptors, containing a BglII restriction site were synthesized as follows: 5'-GATCTCTGTTCTC-3'; 3'-GCAGACAAGACA-3'. The sequence of the insert was verified by restriction mapping of the PCR-generated open reading frame and by limited deoxynucleotide sequencing of the double-stranded cDNA at 3' and 5' ends.

A PCR-based strategy was adopted to subclone the Ca-CC open reading frame into the COS cell expression vector pMT3 (a kind gift of Dr. W. Miller, Brandeis University, Waltham, MA). The COS cell expression vector was constructed by ligating an EcoRI-BamHI fragment of the Ca-CC coding region encompassing either an EcoRI site and a Kozak start translation sequence (5'-GG\textsuperscript{+}ATTTCCGCCGCGAAAATAGTTGGCTCTGTCGACT-3') or a NotI site (5’-ATAGTTGGACGCGCGGATCTTTAACAATATGAATATAACATCT-3'), were used to amplify a PCR reaction run under identical conditions to those described above. The sequence of the insert cloned into pMT3 was verified by restriction mapping and limited deoxynucleotide sequencing at the 3' and 5' ends. Ligation with T\textsubscript{4} ligase and transformation into XLI-Blue were all as described previously (22).

In Vitro Transcription and Translation—cRNA was transcribed from the oocyte cDNA vector expressing the Ribomax kit from Promega. Approximately 25 \mu g of the pGEM11zf\textsuperscript{+} vector containing the cDNA insert was linearized with SalI and transcribed in vitro. The transcription reaction was performed in the presence of a methylguanine cap structure (\textsuperscript{5}'-GppppG\textsuperscript{5}G', 2.4 mM final concentration), to enhance transcript stability. The transcript was transcribed for 4 h at 37°C. The transcript was then linearized with RNaseH and electrophoresed through a 1% agarose/formaldehyde denaturing gel. Antisense cDNA was in vitro transcribed from a eukaryotic expression vector, pCDNA 1, into which the insert had been subcloned using conventional techniques (20). This vector lacked the 3'- and 5'-flanking regions of the Xenopus \beta-globin gene. The vector was linearized with FspI and in vitro transcribed with SP6. The antisense transcript was then synthesized with a methylguaninase cap structure. Transcribed sense cRNA (2 \mu g) was in vitro translated at 30°C for 60 min using rabbit reticulocyte lysate pretreated with microcystic nucleo-
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**Fig. 1. Nucleotide and translated amino acid sequence of Ca-CC cDNA.** The full-length cDNA clone is 3001 bp, of which 2712 bp comprise the open reading frame as indicated by uppercase letters. The polypeptide sequence of 903 amino acids is given in single-letter code underneath the nucleotide sequence. Potential transmembrane domains are underlined. There are potential phosphorylation sites for protein kinase C (13 sites, *●*), Ca2+-calmodulin protein kinase II (10 sites, *●*), and protein kinase A (2 sites, *○*). In addition, there are 12 consensus sites for N-linked glycosylation (**) and for phosphorylation by tyrosine kinase (3 sites, *●*).

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**Expression in Xenopus Oocytes—** Xenopus oocytes were removed and digested by collagenase digestion as described previously (22). Twenty-four hours after defolliculation, stage V/VI oocytes were injected with either 50 nl of nuclease-free water, or 50 nl of water containing either 25 ng sense or 25 ng sense antisense cRNA as described previously (25). Twenty-four hours after defolliculation, stage V/VI oocytes were injected as described previously (22). Expression in Xenopus Oocytes—Xenopus oocytes were removed and digested by collagenase digestion as described previously (22). Twenty-four hours after defolliculation, stage V/VI oocytes were injected with either 50 nl of nuclease-free water, or 50 nl of water containing either 25 ng sense or 25 ng sense + 25 ng antisense cDNA. Recording was performed 2 days postinjection in ND-96 Ringer’s solution (96 mM NaCl, 2.4 mM KCl, 2 mM CaCl2, 1.8 mM MgCl2, 5 mM HEPES, pH 7.4), as described previously (22). In some experiments, membrane vesicles were made from oocytes injected with either water or Ca-CC sense cDNA as described previously (25). Vesicles were subsequently fused to the lipid bilayer for physiological recording.

**Transfection of COS-7 Cells and Whole-cell Patch Clamp Recording—** COS-7 cells were grown to approximately 80% confluency in Dulbecco’s modified Eagle’s medium containing 2 ml -l-glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum at 37°C. Cells were transfected using either chloroquine/dextran (26) or Lipofectamine (Life Technologies, Inc.). We had initially subcloned the Ca-CC open reading frame into two eukaryotic expression vectors, pcDNA1 (Invitrogen) and pMT3 (Promega) in the presence of 0.8 mCi/ml Tran35S-label (ICN). Ca2+-calmodulin protein kinase II (10 sites, ●), Ca2+-calmodulin protein kinase I (10 sites, ○), Ca2+-calmodulin protein kinase I (10 sites, ●), and protein kinase A (2 sites, ○). In addition, there are 12 consensus sites for N-linked glycosylation (**) and for phosphorylation by tyrosine kinase (3 sites, ●).

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**Immunoblot Analysis—** A fusion protein was generated by the Center for AIDS Research Core Facility at the University of Alabama Birmingham from a 570-bp BamHI fragment of the Ca-CC cDNA clone (nucleotides 1170–1740), using the pET21a/T7 expression system (Novagen) for AIDS Research Core Facility at the University of Alabama Birmingham. COS-7 cells were transfected using 20 μg of pMT3-Ca-CC/100-mm dish, containing 10% fetal bovine serum, 1% antibiotics (containing 10% fetal bovine serum, 1% antibiotics) for 2 days prior to transfection and then shocked for 3 min with 10% Me2SO. Cells were incubated with 100 μM chloroquine alone for a further 2 h before being allowed to recover for 2 days in normal medium + 10% fetal bovine serum, 1% penicillin/streptomycin. In the case of transfection with Lipofectamine, 5 μg of plasmid + 25 μg of lipid were added to 500 μl of Dulbecco’s modified Eagle’s medium without further modification of the antigen. Immunoblot analysis of cation-exchange-purified bovine tracheal membrane vesicles was performed as described previously (15). Whole-cell patch clamp recording was carried out using standard techniques. Briefly, the cells were gently scraped, washed in serum-free medium, and added to a chamber placed on the stage of an inverted microscope. The pipette solution contained 112 mM N-methyl-D-glucamine-Cl, 1 mM EGTA, 0.366 mM CaCl2, 2 mM MgCl2, 5 mM HEPES, pH 7.2. The bath solution contained 112 mM N-methyl-D-glucamine-Cl, 30 mM sucrose, 1 mM CaCl2, 2 mM MgCl2, 5 mM HEPES, pH 7.2. Thus, chloride was the main conductive ion. All experiments were performed at room temperature (24 ± 2°C). Filled pipettes were fitted to a suction line and an EPC-7 (List Electronics, Darmstadt, Germany) patch clamp amplifier headstage. Liquid junction potentials were compensated and checked for stability on immersion of the pipette into the bath. The patch pipette was placed onto the cell, and suction was applied until a seal resistance of > 5 GΩ was achieved. After the seal was formed, a sharp suction pulse was applied to form the whole-cell configuration. The membrane potential was clamped at 0 mV and pulsed between ± 100 mV in 20-mV increments at 500-ms intervals under computer control.
control. Currents were digitally recorded and filed for later analysis using the pCLAMP program. Seal resistances were assessed at the conclusion of each experiment to ensure the stability of each preparation.

Tracheal Cl− Channel: Incorporation into Planar Lipid Bilayers—Tracheal chloride channel protein was immunopurified from solubilized bovine tracheal membrane vesicles as described previously (15), using a polyclonal rabbit antibody generated against the 38-kDa form of the protein. The purified protein was incorporated into liposomes as described previously (16) and incorporated into planar lipid bilayer membranes (composed of a mixture of diphytanoyl phosphatidylethanolamine/diphtanyloyl phosphatidylserine/oxidized cholesterol (20 mg/ml) in a 2:1:2 (w/w/w) ratio), in the presence of a symmetrical solution of 100 mM KCl, 10 mM EGTA, and 10 mM MOPS (pH 7). Data analysis was performed as described previously (16).

RESULTS

A polyclonal antibody (p38), raised against a putative Cl− channel protein isolated from bovine trachea (15), was used to screen approximately 10^6 clones in a bovine cDNA expression library. Nine positives were identified, purified by three additional rounds of screening, and sequenced. The inserts were excised from pBluescript with Xhol and NotI. Dideoxy sequencing of the double-stranded DNA revealed that all of the clones were derived from a single mRNA species but differed in size. The full-length sequence (Fig. 1) consists of 3001 bases, of which 255 bases comprise a 3′-untranslated sequence followed by a poly(A)^+ tail. A typical polyadenylation signal sequence (AATAAA), found at bases 13–18 upstream of the poly(A)^+ tail, suggests that this is not an internally A-rich region. Although we did not locate an upstream stop codon, the ATG at position 19 conforms to a conserved Kozak sequence, with an A at the −3 position and a G at the +4 position. One long reading frame extends from base 19 of the full sequence (ATG) that encodes methionine and extends to a TAA stop codon at base 2730, coding for a 903-amino acid protein and predicting a primary translation product of 100 kDa. Motif analysis of the predicted amino acid sequence predicts consensus phosphorylation sites for protein kinase A (2 sites), protein kinase C (15 sites), Ca^2+/-calmodulin-dependent protein kinase (10 sites), and tyrosine kinase (3 sites). In addition, 12 potential sites for N-linked glycosylation are present. This clone does not show any significant homology to any other sequence in the GenBank^1^ database, including the CIC family of chloride channels and p64 (5–9, 11). Hydrophathy analysis of the full-length Ca-CC open reading frame using an analysis window of 19 residues, revealed four major potential transmembrane-spanning domains, as well as several minor regions, consistent with a membrane protein (Fig. 2).

In order to determine if the full-length message from which the clones were derived was isolated, Northern blot analysis of bovine tracheal mRNA was carried out under high stringency conditions. As shown in Fig. 3A, a positively hybridized mRNA signal was detected at 3.1 kilobases, suggesting that close to a full-length sequence was cloned. In addition, RT-PCR analysis of mRNA from five different bovine tissues (trachea, lung, liver, brain, and renal papilla) and from Xenopus oocytes was performed to determine if the cloned cDNA was exclusively expressed in bovine trachea. As shown in Fig. 3B, following 30 cycles of PCR, signal of the predicted size (704 bp) was detected only in RNA extracted from the trachea. The appropriate genomic controls were also negative. All other bovine tissues examined, as well as RNA from Xenopus oocytes, were negative using these primers, although the integrity of the RNA from these samples was maintained, as evidenced by the amplification of a 150-bp fragment of bovine β-actin from the bovine samples and a 650-bp product using human β-actin primers from Xenopus RNA. Following an additional 30 cycles of PCR, RNA samples that had been reverse-transcribed with avian myeloblastosis virus-reverse transcriptase remained negative (results not shown). Although only detected in the trachea among the bovine tissues tested, the Ca-CC clone did cross-hybridize with DNA from mouse, rat, dog, rabbit, monkey, and human, as shown in Fig. 3C, suggesting that the clone is conserved between species.

In Vitro Transcription and Translation—In order to determine whether the tracheal cdNA could encode a functional chloride channel protein, the cdNA was subcloned into an oocyte expression vector. This vector was a modified form of pGEM11zf− and included the 5′- and 3′-untranslated region of the Xenopus β-globin gene flanking the coding region of the tracheal cdNA insert. The Ca-CC insert was modified to include BglII restriction sites 3′ and 5′ to the coding region, to facilitate subcloning into the vector. Sense cdRNA for injection into Xenopus oocytes was in vitro transcribed from the vector using the T7 promoter. In vitro translation of the cdRNA yielded a major polypeptide product that migrated at 100 kDa on SDS-PAGE (Fig. 4). In the presence of canine pancreatic microsomes, the Mr of the polypeptide shifted to 140,000. Several smaller protein bands, presumably equivalent to partial transcripts were also detected by autoradiography. Neither the 100- nor 140-kDa polypeptide products were reduced to smaller forms by treatment with 50 mM DTT. Antisense cdRNA, without
the Xenopus β-globin-flanking regions, was in vitro transcribed from a separate vector (pcDNA I), using the SP6 promoter.

Immunoblot Analysis—In order to further characterize the relationship between the cloned Ca-CC and the native bovine tracheal protein, we performed an immunoblot analysis using a rabbit polyclonal antibody raised against a fusion protein generated from the Ca-CC cDNA sequence to probe tracheal proteins partially purified by cation exchange of solubilized bovine tracheal material, consistent with our earlier observations (15). Similarly, the polyclonal antibody generated against the Ca-CC fusion protein also recognized a polypeptide migrating with an Mr of 38,000 in the cation exchange-purified tracheal material, suggesting that both the native tracheal anion channel and the Ca-CC protein share a high degree of immunological identity.

Expression in Xenopus Oocytes—Expression studies were performed in stage V/VI oocytes isolated from Xenopus laevis and defolliculated by collagenase digestion. Oocytes were injected with water, 50 ng of sense, or 25 ng of sense or 125 ng of antisense cRNA and then incubated at 18°C for 48 h prior to recording. Whereas the whole-cell current records of oocytes injected with sense or antisense cRNA were indistinguishable from records made from water-injected oocytes, oocytes injected with sense cRNA alone displayed an increased current at every potential in the absence of calcium ionophore (Fig. 6). At a holding potential of −100 mV, the mean current (in pA ± S.E.) was −96 ± 25 (n = 14), in water-injected oocytes, −157 ± 46 (n = 7) in sense + antisense-injected oocytes, and −2052 ± 528 (n = 8) in oocytes injected with Ca-CC sense cRNA. At a holding potential of +80 mV, the mean current (in pA ± S.E.) for water-injected oocytes was 457 ± 209 (n = 14); for sense + antisense-injected oocytes, 847 ± 278 (n = 7); and for sense-injected oocytes, 8084 ± 1700 (n = 8). The I/V curve (Fig. 7) was outwardly rectified at higher positive voltages and reversed close to 0 mV, consistent with an anion-selective channel. Fur-
have expressed cRNAs of either epithelial Na\(^{+}\) current or sense or antisense cRNA-injected Xenopus oocytes. Oocytes were injected with water, 50 ng of sense, or 25 ng of sense + 25 ng of antisense Ca-CC cRNA. The holding potential was stepped in 20-mV increments at 500-ms intervals from -100 mV to +80 mV.

Further elevation of [Ca\(^{2+}\)] by 1 \(\mu\)M ionomycin resulted in activation of the endogenous Ca\(^{2+}\) -activated Cl\(^{-}\) current that could be blocked by niflumic acid (results not shown).

The expressed current was also sensitive to 100 \(\mu\)M DIDS (Fig. 8A) and to 1 mM DTT (Fig. 8B), consistent with our previous observations of an anion-selective channel isolated from bovine trachea (16). DIDS, at a holding potential of -100 mV, decreased the whole-cell current to 61 ± 8% (S.E.) of the pre-DIDS control value, and to 55 ± 7% at a holding potential of +80 mV (n = 4). The reducing agent DTT decreased whole-cell current at -100 mV and +80 mV to 73 ± 6% and 78 ± 6% of control, respectively (n = 5). In other experiments where we have expressed cRNAs of either epithelial Na\(^{+}\) channels or CFTR in oocytes, neither DTT nor DIDS had any effect on the magnitude of the observed currents (results not shown).

In contrast, in three separate experiments, the expressed current was not sensitive to 100 \(\mu\)M niflumic acid (Fig. 9A), a compound previously identified to inhibit the endogenous Ca\(^{2+}\) -activated Cl\(^{-}\) channel of Xenopus oocytes (18). In the oocyte, the apparent \(K_{t}\), for niflumic acid inhibition of the endogenous Ca\(^{2+}\) -activated Cl\(^{-}\) channel is 17 \(\mu\)M (18). Similarly, the tracheal anion channel, when reconstituted into planar lipid bilayers, was also insensitive to this compound (Fig. 9B). At a holding potential of +40 mV under control conditions, the \(P_{o}\) of the bovine tracheal Ca-CC incorporated into the lipid bilayer was 0.38 ± 0.04 (n = 9). Following the addition of 100 \(\mu\)M niflumic acid to both the cis and trans sides of the bilayer, the \(P_{o}\) remained at 0.36 ± 0.07 (n = 4). Furthermore, and consistent with our previous observations (17), this channel was apparently insensitive to a \(\alpha\)M elevating mixture of 10 \(\mu\)M forskolin and 1 mM isobutylmethylxanthine when expressed in Xenopus oocytes (data not shown).

Whole-cell Patch Clamp Recordings in Ca-CC Transfected COS-7 Cells—In addition to examining Ca-CC expression in Xenopus oocytes, we have also expressed the Ca-CC clone in the simian renal cell line, COS-7. As judged visually by light microscopic inspection of cells transfected with a vector containing the lacZ gene behind the CMV promoter (pCMV\(_{\text{gal}}\)), a kind gift of Dr. E. Sorscher, Gregory Fleming James Cystic Fibrosis Research Center, University of Alabama at Birmingham) under identical conditions to those used for the transfection of the Ca-CC clone and developed with 5-bromo-4-chloro-3-indoyl \(\beta\)-D-galactoside (Life Technologies, Inc.), at least 30% of the cells were transfected. Consistent with this observation, out of 11 transfected cells patched, 9 showed ionomycin-sensitive increases in whole-cell chloride current. In contrast, in five non-transfected cells (either control or mock-transfected), we never observed activation of ionomycin-sensitive currents. The \(\chi^2\) value for these observations was 6.32, suggesting that the difference in frequency was highly significant (0.02 > p > 0.01). As shown in Fig. 10, A and B, chloride currents in either the control nor the mock-transfected cells were affected by 2 \(\mu\)M ionomycin. In contrast, the transfected cells exhibited increased currents in the presence of the calcium ionophore (Fig. 10C). The increased current could be reversed to near preionophore levels by a 20-min washout. Fig. 11 illustrates the whole-cell current/voltage relationships before and after cell exposure to ionomycin together with the appropriate difference currents in transfected cells (panel B) and in control cells (panel A). Perfusion of the reducing agent DTT (4 mM) through the bath following the addition of ionophore, completely abolished the increase in current due to ionomycin in transfected cells (Fig. 11C). Under whole-cell conditions, the I/V curve was linear and reversed at 0 mV (Fig. 12). Seal resistances were >1 G\(\Omega\). Therefore, the leak (which also reverses at 0 mV) was small as seen in the preionomycin currents.

Planar Bilayer Recording of Vesicles Prepared from Ca-CC-expressing Oocytes—In order to characterize further the Ca-CC channel, we prepared membrane vesicles from oocytes injected with Ca-CC cRNA and fused these vesicles to the lipid bilayer. As shown in Fig. 13, the channel observed was insensitive to niflumic acid (100 \(\mu\)M), as previously determined for the native channel biochemically isolated from the tracheal epithelium (Fig. 8B). Under control conditions (100 mM symmetrical KCl), the \(P_{o}\) was 0.41 ± 0.07 (mean ± S.D., n = 24), and the single channel conductance was 21 picoamperes. In the presence of 100 \(\mu\)M niflumic acid, the \(P_{o}\) was 0.42 ± 0.06 (n = 24). However, the channel could be activated by increasing [Ca\(^{2+}\)], but only from one side of the bilayer, (i.e. the side opposite from which
DIDS inhibits); in the presence of 10 μM Ca$^{2+}$, the $P_o$ increased to 0.60 ± 0.08 ($n = 24$). These results agree well with our previous observations of the Ca$^{2+}$-activated chloride channel isolated from the bovine trachea, which, in the absence of phosphorylation, exhibited a $P_o$ of 0.55 in the presence of 10 μM Ca$^{2+}$ (17). The addition of either 10 μM DTT or 100 μM DIDS reduced the observed $P_o$ of the Ca$^{2+}$-activated channel to 0.15 ± 0.06 ($n = 6$) and 0.06 ± 0.02 ($n = 5$), respectively. Using membrane vesicles isolated from Ca-CC-expressing oocytes, we were also able to determine the ion selectivity of the channel. The channel was both anion-selective ($P_{A^{-}} : P_{Cl^{-}} = 8:1$, $n = 7$, $\phi_{rev} = 53 ± 2$ mV), and selective for iodide over chloride ($P_{I^{-}} : P_{Cl^{-}} = 3:1$, $n = 4$, $\phi_{rev} = 27 ± 2$ mV), as determined under biionic conditions. These findings are also consistent with our previ-
viously reported observations for a native Ca\(^{2+}\)-activated chloride channel purified from the bovine trachea, which exhibited a single channel conductance of 25–30 picosiemens (in symmetrical 150 mM KCl) and showed an I\(^{-} \rightarrow \) Cl\(^{-}\) anion selectivity of 2.1:1 (16, 17).

In approximately one out of every five vesicle incorporations, we observed a channel of similar conductance to the Ca-CC that was also activated by Ca\(^{2+}\). However, this channel was in all cases sensitive to niflumic acid (included routinely in all experiments) and was observed in vesicles isolated from water-injected oocytes. The sensitivity of the channel to niflumic acid suggests that the vesicles contained the Ca\(^{2+}\)-activated Cl\(^{-}\) conductance endogenous to the oocyte.

**DISCUSSION**

We have previously described the purification and electrophysiological characteristics of an epithelial anion channel isolated from the bovine trachea. Prominent among the channel properties exhibited by this protein are its activation by calcium-mediated agonists (as opposed to cAMP-mediated agonists) and its sensitivity to the reducing agent dithiothreitol (15, 17). Using an antibody raised against this protein to screen a bovine tracheal cDNA expression library, we have isolated a candidate cDNA clone. Many of the features exhibited by the cDNA sequence are consistent with a membrane protein regulated by calcium rather than by protein kinase A, e.g. consensus phosphorylation sequences for protein kinase C and calmodulin-dependent protein kinase II, but few for protein kinase A, and consensus sites for N-linked glycosylation. Furthermore, expression of the full-length cDNA in either Xenopus oocytes or mammalian COS-7 cells is associated with the appearance of a Ca\(^{2+}\)-activated anion channel that shares many of the features previously associated with the anion channel purified from the bovine trachea, e.g. activation by calcium, sensitivity to DTT and DIDS, and insensitivity to niflumic acid, even at concentrations 5 times the apparent K\(_{i}\) of this compound in the oocyte (18). In addition, when the cDNA was expressed in COS-7 cells, the protein exhibited a linear I/V relationship, a characteristic also previously attributed to the purified bovine channel protein when it was incorporated into planar lipid bilayers. In contrast, when expressed in Xenopus oocytes, the I/V curve for the Ca-CC channel was found to be outwardly rectified. This phenomenon has, however, also been observed when CFTR, which also behaves as a linear anion channel when incorporated into planar lipid bilayers or studied under patch clamp recording conditions (1, 27), is expressed in Xenopus oocytes (28).

Although the channel expressed by the Ca-CC cDNA clone and the anion channel protein purified from the bovine trachea have many features in common as assessed functionally, one important divergence resides in the different biochemical behaviors of the purified protein as compared with that translated in vitro from the Ca-CC clone. We have previously demonstrated that the anion channel protein purified from the bovine trachea is in its native state a 140-kDa protein and in this form acts as a functional channel when incorporated into planar lipid bilayers (16). When subjected to chemical reduction by the reducing agent DTT, the mobility of the purified protein on SDS-PAGE shifts to 36–38 kDa, although an intermediate form migrating at 60–64 kDa can also be observed (15, 16). In the reduced form, the protein no longer forms a func-
tional ion channel. Furthermore, ion channel activity associated with the 140-kDa protein incorporated into planar bilayers is markedly inhibited when DTT is added to the bilayer (16). In contrast, the polypeptide product translated from the Ca-CC cDNA expression library that encodes a protein that behaves as a Ca\(^{2+}\)-activated anion channel in oocytes or in mammalian COS-7 cells. Importantly, this newly identified channel shares little homology (less than 40%), with the CIC family of voltage-sensitive anion channels identified by Jentsch et al. (5–7) and, based on limited RT-PCR analysis of the cDNA and sensitivity of the expressed channel to niflumic acid, is separate from the Ca\(^{2+}\) selective channel previously identified in bovine tracheal membrane vesicles (15, 16). This channel may thus provide a valuable alternative pathway for pharmacological manipulation of chloride secretion in cystic fibrosis.

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Fig. 12. Whole-cell I/V curve in Ca-CC-transfected COS-7 cells in the presence of ionomycin. Under whole-cell conditions, in the presence of 2 μM ionomycin, the I/V curve of pMT3-Ca-CC-transfected cells was linear, and it reversed close to 0 mV.

Fig. 13. Effect of Ca\(^{2+}\) and blockers on oocyte-expressed Ca-CC channels incorporated into planar lipid bilayers. Membrane vesicles were prepared from Xenopus oocytes injected with Ca-CC cRNA and fused to the planar lipid bilayer. The observed channel was insensitive to niflumic acid (100 μM), but could be activated by 10 μM Ca\(^{2+}\). Ca-CC channel open probability was significantly decreased by both DTT (10 μM) and DIDS (100 μM). Holding potential was +60 mV. Dashed line represents zero current level in all cases. Records were filtered at 200 Hz.
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