

Functional effects of novel anti-ClC-3 antibodies on native volume-sensitive osmolyte and anion channels in cardiac and smooth muscle cells

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Wang, Ge-Xin, William J. Hatton, Grace L. Wang, Juming Zhong, Ilia Yamboliev, Dayue Duan, and Joseph R. Hume. Functional effects of novel anti-ClC-3 antibodies on native volume-sensitive osmolyte and anion channels in cardiac and smooth muscle cells. *Am J Physiol Heart Circ Physiol* 285: H1453–H1463, 2003. First published June 19, 2003; 10.1152/ajpheart.00244.2003.—Whether ClC-3 encodes volume-sensitive organic osmolyte and anion channels (VSOACs) remains controversial. We have shown previously that native VSOACs in some cardiac and vascular myocytes were blocked by a commercial anti-ClC-3 carboxy terminal antibody (Alm C_{592–661} antibody), although recent studies have raised questions related to the specificity of Alm C_{592–661} antibody. Therefore, we have developed three new anti-ClC-3 antibodies and investigated their functional effects on native VSOACs in freshly isolated canine pulmonary artery smooth muscle cells (PASMCs) and guinea pig cardiac myocytes. These new antibodies produced a common prominent immunoreactive band with an apparent molecular mass of 90–92 kDa in the guinea pig heart and PASMCs, and a similar molecular mass immunoreactive band was observed in the brain from homozygous Clcn3^{+/+} mice but not from homozygous Clcn3^{-/-} mice. VSOACs elicited by hypotonic cell swelling in PASMCs and guinea pig atrial myocytes were nearly completely abolished by intracellular dialysis with two new anti-ClC-3 antibodies specifically targeting the ClC-3 carboxy (C_{670–687} antibody) and amino terminus (A_{1–14} antibody). This inhibition of native VSOACs can be attributed to a specific interaction with endogenous ClC-3, because 1) preabsorption of the antibodies with corresponding antigens prevented the inhibitory effects, 2) extracellular application of a new antibody raised against an extracellular epitope (E_{X133–148}) of ClC-3 failed to inhibit native VSOACs in PASMCs, 3) intracellular dialysis with an antibody targeting Kv1.1 potassium channels failed to inhibit native VSOACs in guinea pig atrial myocytes, and 4) anti-ClC-3 C_{670–687} antibody had no effects on swelling-induced augmentation of the slow component of the delayed rectifying potassium current in guinea pig ventricular myocytes, although VSOACs in the same cells were inhibited by the antibody. These results confirm that endogenous ClC-3 is an essential molecular entity responsible for native VSOACs in PASMCs and guinea pig cardiac myocytes.

chloride channels; cell volume

PREVIOUS EVIDENCE suggested a role for ClC-3 as a molecular candidate for native volume-sensitive organic osmolyte and anion channels (VSOACs) (28) in some types of mammalian cells. This evidence included 1) demonstration that stable or transient transfection of ClC-3 cDNA, cloned from a guinea pig cardiac ventricle (short isoform gpClC-3), into NIH/3T3 cells yielded basally active outwardly rectifying chloride currents that were strongly modulated by cell volume and exhibited many properties similar to native VSOACs, including an anion selectivity of $I^- > Cl^-$ (7); 2) site-directed mutagenesis altered rectification and anion selectivity (7) and regulation by cell volume and protein kinase C of the expressed gpClC-3 current ($I_{gpClC-3}$) (5); and 3) native VSOACs in cardiac and smooth muscle cells and *Xenopus* oocytes, as well as expressed $I_{gpClC-3}$, were found to be significantly inhibited by intracellular dialysis of a commercially available anti-ClC-3 antibody (8). Subsequent reports (15, 20) initially confirmed that expression of human ClC-3 (hClC-3) yielded outwardly rectifying chloride currents that were strongly modulated by cell volume (however, see Ref. 34), and native VSOACs in bovine epithelial cells (33), HeLa cells, and *Xenopus* oocytes (14) were found to be significantly inhibited by ClC-3 antisense. Consistent with the proposed role of VSOACs in regulatory cell volume homeostasis (28), in these studies regulatory volume decreases were also shown to be attenuated in cells treated with ClC-3 antisense.

Despite this evidence, a series of more recent studies has failed to support a role of ClC-3 in cell volume regulation (reviewed in Refs. 12, 17, and 19). Certainly, in some of these studies, failure to identify recordable currents due to expression of ClC-3 in various heterologous expression systems can be attributed to the difficulty of distinguishing membrane currents due to transgenic expression from endogenous Cl^- currents present in most cell types. A similar inability to distinguish currents due to transgenic expression from native Cl^- currents likely contributed to earlier erroneous conclusions regarding the role of P-glycoprotein and pICln as molecular candidates for VSOACs (3, 29).

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It has recently been suggested from studies of *Clcn^{-/-}* mice that CLC-3 may play a major role in the endosomal compartment of neurons and be partially responsible for acidification of synaptic vesicles (27). A novel splice variant CLC-3B with a consensus motif for binding PDZ domains, when coexpressed with an epithelium-specific scaffolding protein, has recently been found to increase the activity of outwardly rectifying anion channels possibly due to a specific interaction with cystic fibrosis transmembrane conductance regulator (CFTR) channels in epithelial cells (22), although a subsequent study failed to confirm such an interaction (11).

The purpose of the present study was to develop several novel anti-CLC-3 polyclonal antibodies and examine whether these functionally alter the properties of native VSOACs expressed in canine pulmonary arterial smooth muscle cells (PASMCs) and guinea pig cardiac myocytes. Although native VSOACs in cardiac and smooth muscle cells were previously found to be significantly inhibited by intracellular dialysis of the commercially available (Alomone Labs) anti-CLC-3 antibody (8), serious questions have been raised regarding the relative specificity of this particular antibody (27, 34). The ability of these newly developed antibodies to functionally alter the properties of native VSOACs in smooth muscle and cardiac myocytes would 1) provide strong evidence supporting a role of endogenous CLC-3 as the protein responsible for native VSOACs in these cell types and 2) provide new experimental tools for future studies to distinguish Cl^- currents due to CLC-3 expression from endogenous Cl^- currents present in heterologous cell expression systems and to test for the possible expression of different subtypes of native VSOACs in a variety of cells. A preliminary report (32) of these results has been published.

METHODS

Immunizing peptide sequence design and synthesis. CLC-3 belongs to the same branch of the CLC family as CLC-4 and CLC-5, which share ~80% identity (31). Attempts to produce CLC-3-specific antibodies therefore require peptide sequences be designed from regions that show the least homology to CLC-4 and CLC-5. Peptide sequences selected for the production of CLC-3-specific antibodies were based on the following criteria: 1) specificity to CLC-3 isoforms, 2) lack of homology to the closely related CLC-4 and CLC-5 proteins, and 3) lack of homology to other cellular proteins. The selected amino acid sequences (see Fig. 1) based on the above criteria were 1) MTNGGSINSSTHLL corresponding to residues 1–14 (14 amino acids) found at the amino terminal end of mouse CLC-3 (short form), the resultant antibody referred to as A_{1-14} antibody; 2) KTWAEIIGQAEQPGS corresponding to residues 133–148 (16 amino acids), a region found in an extracellular portion between transmembrane domains D1 and D2 of mouse CLC-3 (short form), the resultant antibody referred to as $Ex_{133-148}$ antibody; and 3) GSSRVCFQAHTPSLPAES corresponding to residues 670–687 (18 amino acids), a region found at the carboxy terminus of mouse CLC-3 (short form), the resultant antibody referred to as $C_{670-687}$ antibody.

The selected amino acid sequences were submitted to Research Genetics (Huntsville, AL) and assessed for suitability

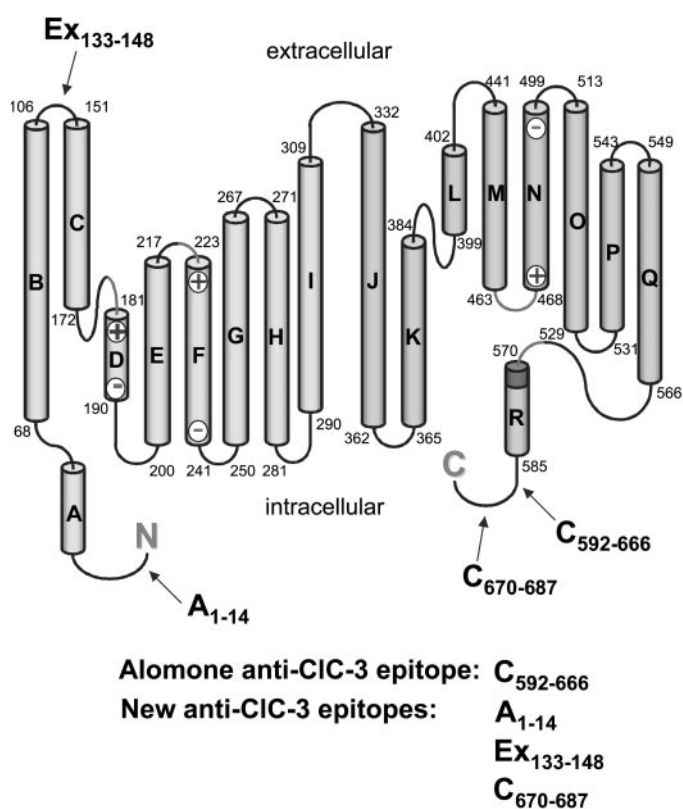


Fig. 1. Mouse CLC-3 (short form) channel topology model adapted from the prokaryotic StCLC crystal structure [adapted from Dutzler et al. (9)] compares the location of the peptide epitope regions of the new anti-CLC-3 antibodies and the commercially available carboxy-terminal Alm antibody. Letters within the tubes refer to the amino acids making up transmembrane helices of the protein, which are likely similar between StCLC and CLC-3 proteins.

as antigenic candidates. This assessment took into consideration the existence of multiple hydrophilic charged residues, the number of neutral residues, balance of charge throughout the sequences, the number of prolines and for excessively long repeats of identical amino acids. All three sequences were deemed to be potentially useful antigenic candidates for antibody production. Peptides were synthesized according to Research Genetics' protocols incorporating multiple antigen peptide resin technology to enhance antigenic response. To ensure the specificity of the newly synthesized peptides for CLC-3, each of the peptide sequences was searched against the SwissProt protein database at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) algorithm (1). Each epitope sequence was found to be 100% identical to CLC-3 from several species, including guinea pigs, humans, rats, mice, and rabbits. In addition, each antibody epitope was subjected to a BLAST search of the protein database (SwissProt) using the computer program blastp with an expect value of 1,000. No significant homology (expect value <1,000) was found to other known proteins [except peptide A_{1-14} , which had some similarity to a trithorax nuclear protein from *Drosophila melanogaster* (expect value = 95)]. Each antibody should detect both the long and short isoforms of CLC-3. Amino acid alignment of each peptide sequence with the closely related CLC-4 and CLC-5 proteins indicated that peptide A_{1-14} had 0 of 14 residues identical to CLC-4 and CLC-5; peptide $Ex_{133-148}$ had 6 of 16 and 5 of 16 residues identical to CLC-4 and CLC-5,

respectively; peptide C₆₇₀₋₆₈₇ had 7 of 18 and 5 of 18 residues identical to CIC-4 and CIC-5, respectively. The peptide epitopes used here to generate A₁₋₁₄ and C₆₇₀₋₆₈₇ antibodies were similar to the peptide epitopes from the human long form CIC-3 previously used to generate specific antibodies that exhibited no cross-immunoreactivity with human CIC-1, rat CIC-2, or human CIC-4 (16).

A commercially available CIC-3 antibody from Alomone Labs (Israel) was also used in this study. This antibody was generated from a long peptide sequence (see Fig. 1) corresponding to carboxy terminus residues 592–661 (70 amino acids), referred to as Alm C₅₉₂₋₆₆₁ antibody.

Immunization and harvesting of antibodies. Animals were immunized with the synthetic linear peptides by Research Genetics according to their established protocols. In brief, each peptide (with a cysteine added to the carboxy terminal) was conjugated to keyhole-limpet hemocyanin conjugate, emulsified with an equal volume of Freud's incomplete adjuvant, and injected into two New Zealand White rabbits. The animals then received a boost after 2 wk, a bleed after 4 wk, a second boost after 6 wk, a second bleed and further boost after 8 wk, and a production bleed at *week 10*. Sera was removed from the *week 10* bleed, and the antipeptide antibody titer was determined by enzyme-linked immunosorbent assay. Immunization and bleeds were maintained for 4 mo. The crude antibodies obtained over this period were then affinity purified by Research Genetics. Briefly, peptide antigen was immobilized on an activated support. Antisera were passed through the sera column and then washed. Specific antibodies were eluted via a pH gradient into neutralizing buffer, collected, and stored in borate buffer (0.125 M total borate, pH 8.2). The borate buffer itself is antimicrobial, and the purified sera was stored at 4°C.

Western blot analysis of antibodies. Pieces of guinea pig ventricle and canine pulmonary artery were dissected, bathed in ice-cold PBS buffer while cleaned from connective tissues, and split into two portions. One portion was glass-glass homogenized in RIPA buffer composed of 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM Na₂EDTA, 0.5% (vol/vol) Nonidet P-40, 0.5% Triton X-100, 0.1% SDS, 1 mM NaF, 1 μM leupeptin, 1 μM AEBSF, and 10% glycerol to produce total protein extracts. The second portions were digested to produce freshly dispersed cells. Cells were centrifuged for 15 min at 14,000 rpm and 4°C, clear supernatant was discarded, and the cells were resuspended in 100 μl RIPA buffer and lysed by 20 passages through a 25-gauge needle. To increase protein yield, tissue and cell homogenates were subjected to four cycles of short sonication, vortexing, and incubation on ice for a total of 1 h. Protein concentrations were assayed by the Micro BCA protein assay (Pierce; Rockford, IL). Equal amounts of total protein (25 μg) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes for 1.5 h at 24 V, 4°C (Genie blotter, Idea Scientific; Minneapolis, MN). The membranes were blocked for 1 h with 5% skim milk in PBS before being probed with antibodies at the following dilutions with 5% milk-PBS: Alm C₅₉₂₋₆₆₁, 1:200; A₁₋₁₄, 1:100; Ex₁₃₃₋₁₄₈, 1:500; and C₆₇₀₋₆₈₇, 1:500. Incubation with the primary antibody solutions took place at room temperature for 2 h or overnight at 4°C. Excess primary antibody was removed by three 5-min washes with 100 mM Tris (pH 7.5), 0.1% Tween 20, and 150 mM NaCl (TNT buffer), followed by a 1-h incubation with a secondary alkaline phosphatase-conjugated antibody (Jackson ImmunoResearch Laboratories; West Grove, PA) diluted 1:10,000 in 5% milk-PBS. Color was developed using the 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP-NBT) alkaline phosphatase

substrate. Blots were scanned with a UMAX Powerlook flatbed scanner (Bio-Rad; Hercules, CA) to obtain images.

A limited number of homozygous Clcn3^{-/-} mice were kindly provided by Dr. Fred Lamb of the University of Iowa (4) for Western blot analysis of CIC-3 in the mouse brain. The tissue was weighed and glass-glass homogenized with 10 vol RIPA buffer, as specified above. After centrifugation at 14,000 rpm for 15 min, the supernatant was transferred into a clean tube, and the membrane-containing pellet was resuspended in 10 vol RIPA buffer with a Teflon pestle. The suspension was vigorously vortexed and boiled for 15 min before CIC-3 protein assay by Western blotting.

Single cell preparation. Single guinea pig atrial and ventricular myocytes and canine PSMCs were enzymatically isolated as previously described (6, 35). Mongrel dogs were euthanized with pentobarbital sodium (45–50 mg/kg iv), whereas guinea pigs were euthanized by CO₂ inhalation. Freshly dispersed cells were used within the same day. All experiments were carried out in accordance with the recommendations of the University of Nevada Animal Care and Use Committee.

Electrophysiological recordings. A drop of cell suspension was added to a recording chamber (volume, 0.4 ml) mounted on an inverted microscope (Nikon Eclipse TS100), and superfused with bath solutions at a rate of 2 ml/min. All the experiments were conducted at room temperature (22–24°C). Membrane currents were measured using tight-seal whole cell voltage-clamp techniques (13). Patch pipettes were made from borosilicate glass capillaries and had a tip resistance of 1.5–2.5 MΩ when filled with pipette solutions. The bath and pipette solutions were connected via Ag/AgCl wires to a patch-clamp amplifier (3900A Integrating Patch Clamp, Dagan; Minneapolis, MN). A 3 M KCl-agar salt bridge between the bath and Ag/AgCl reference electrode was used to minimize changes in liquid junction potential. Activation of VSOACs was achieved by swelling the cells with hypotonic bath solution. To follow the time course of changes in membrane currents, repetitive voltage-clamp steps were applied every 30 s from a holding potential of –40 mV. The membrane potential was first hyperpolarized to –80 mV for 100 ms, then clamped to 0 mV for 10 ms, and subsequently depolarized to +80 mV for 100 ms. Current-voltage relations were obtained by measuring the membrane currents elicited by 400-ms pulses to potentials ranging from –100 to +120 mV in 20-mV increments applied from a holding potential of –40 mV every 2 s. For simultaneous measurement of VSOACs and the slow component of the delayed rectifying potassium current (*I*_{Ks}), 2-s depolarizing pulses to potentials ranging from –20 to +80 mV in 20-mV increments from a holding potential of –40 mV were applied every 5 s. Membrane currents were filtered at a frequency of 1 kHz and digitized on-line at 5 kHz using a Pentium III processor and pCLAMP 8 software (Axon Instruments; Foster City, CA). Current amplitudes of VSOACs were measured at 10 ms after the corresponding voltage steps, and *I*_{Ks} amplitudes were obtained at the end of depolarizing test pulses.

Solutions and reagents. For VSOAC current recording, the bath and pipette solutions were chosen to avoid contamination of other ionic currents. Symmetrical chloride concentration was used for the bath and pipette solutions. For PSMC, the hypotonic (230 mosmol/kg) bath solution contained (in mM) 107 N-methyl-D-glucamine chloride (NMDG-Cl), 1.5 MgCl₂, 2.5 MnCl₂, 0.5 CdCl₂, 0.05 GdCl₃, 10 glucose, and 10 HEPES; pH = 7.4 adjusted with NMDG. The isotonic (310 mosmol/kg) and hypertonic (350 mosmol/kg) solutions contained additional 80 and 120 mM mannitol, respectively. The pipette solution contained (in mM) 96 CsCl, 20 TEA-Cl, 5

ATP-Mg, 5 EGTA, 80 mannitol, and 5 HEPES; pH = 7.2 adjusted with CsCl (300 mosmol/kg adjusted with mannitol). For guinea pig atrial myocytes, the hypotonic (220 mosmol/kg) bath solution contained (in mM) 90 NaCl, 0.8 MgCl₂, 1.0 CaCl₂, 0.2 CdCl₂, 2.0 BaCl₂, 10 TEA-Cl, 10 HEPES, and 5.5 glucose; pH = 7.4 adjusted with NaOH. The isotonic (300 mosmol/kg) and hypertonic (350 mosmol/kg) bath solutions contained additional 80 and 130 mM mannitol, respectively. The pipette solution was composed of (in mM) 108 NMDG-Cl, 2 EGTA, 5 Mg-ATP, and 10 HEPES; pH = 7.4 adjusted with NMDG (290 mosmol/kg adjusted with mannitol). For simultaneous measurement of VSOACs and *I*_{Ks}, the hypotonic (220 mosmol/kg) bath solution contained (in mM) 95 NaCl, 5 KCl, 1 CaCl₂, 0.8 MgCl₂, 2 BaCl₂, 0.2 CdCl₂, 0.33 NaH₂PO₄, 10 HEPES, and 5.5 glucose; pH = 7.4. The isotonic solution (300 mosmol/kg) contained additional 80 mM mannitol. The pipette solution consisted of (in mM) 108 KCl, 5 Mg-ATP, 2 EGTA, and 10 HEPES; pH = 7.4 (290 mosmol/kg adjusted with mannitol). In all cases, the osmolality of the pipette solution was 10 mosmol/kg lower than the corresponding isotonic bath solutions to avoid spontaneous cell swelling.

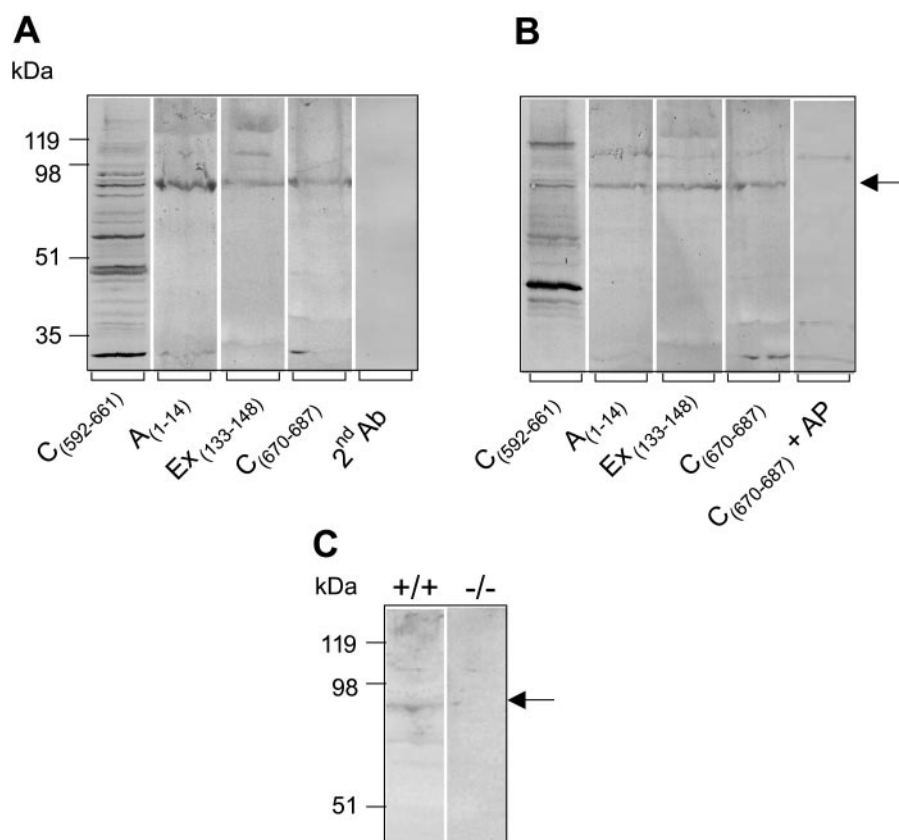
Intracellular dialysis with anti-CLC-3 antibodies (antibodies) was performed as previously described (8). Antibodies were added to the pipette or bath solutions depending on the types of antibodies being used at final concentrations as indicated. For the preparation of antigen-preabsorbed antibodies, antigen and antibodies were mixed in a molar ratio of 10:1, stored at 4°C overnight, and added to the pipette solution to achieve final concentrations. The osmolality of the pipette dialysis solution was not altered by inclusion of either antibody alone or preabsorbed antibody. In Figs. 3–6, the onset of membrane rupture and intracellular dialysis is indicated at *time 0*.

Data analysis. Data are expressed as means ± SE with *n* representing the number of cells. Statistical analyses were made by Student's two-tailed paired *t*-test and one-way repeated-measures analysis of variance (Student-Newman-Keuls method) where appropriate. Probability (*P*) values of <0.05 were considered statistically significant.

RESULTS

Western blot analysis of anti-CLC-3 antibodies. Consistent with the results of our earlier study (2) of hearts from guinea pigs, canines, and rats, Western blot analysis using the commercial Alm C₅₉₂₋₆₆₁ antibody revealed numerous immunoreactive bands in the guinea pig heart and canine PAMSCs (Fig. 2, *A* and *B*). In contrast, the three new anti-CLC-3 antibodies (A₁₋₁₄, Ex₁₃₃₋₁₄₈, and C₆₇₀₋₆₈₇) produced a common prominent immunoreactive band with an apparent molecular mass of 90–92 kDa in the guinea pig heart and PAMSCs. A similar molecular mass immunoreactive band was also observed using the C₆₇₀₋₆₈₇ antibody in the brains from homozygous Clcn3^{+/+} mice but not from homozygous Clcn3^{-/-} mice (Fig. 2*C*), suggesting this immunoreactive 90- to 92-kDa signal indeed represents CLC-3 protein. Despite the apparent lack of specificity observed with the commercial Alm C₅₉₂₋₆₆₁ antibody, a similar 90- to 92-kDa immunoreactive band, as observed with antibodies A₁₋₁₄, Ex₁₃₃₋₁₄₈, and C₆₇₀₋₆₈₇, could be observed with this antibody as well. Some relatively minor immunoreactivity was observed at a higher and lower molecular weights using the

Fig. 2. Western blot analysis of anti-CLC-3 antibodies in the guinea pig (GP, *A*) heart, canine pulmonary arterial smooth muscle cells (PAMSCs, *B*), and the mouse brain (*C*). A common prominent immunoreactive band (arrow) with an apparent molecular mass of 90–92 kDa was observed with all four antibodies in GP heart (*A*) and PAMSCs (*B*). A similar molecular mass immunoreactive band was observed using the C₆₇₀₋₆₈₇ antibody (Ab) in the brain from homozygous Clcn3^{+/+} mice but not from homozygous Clcn3^{-/-} mice (*C*), suggesting the immunoreactive 90- to 92-kDa signal is specific for CLC-3 protein. The CLC-3 immunoreactive signal was not observed in control blots using the secondary antibody alone in the GP heart or PAMSCs or with the antigen-preabsorbed (AP) C₆₇₀₋₆₈₇ antibody in PAMSCs (C₆₇₀₋₆₈₇ antibody + AP).



three new anti-CLC-3 antibodies, which was likely non-specific because it was still present when the antibody was preabsorbed with antigen (Fig. 2B, lane 5).

Intracellular dialysis of anti CLC-3 C₆₇₀₋₆₈₇ and A₁₋₁₄ antibodies blocks native VSOACs in canine PSMCs. Figure 3 illustrates the effects of intracellular dialysis with a new anti-CLC-3 carboxy terminus antibody (C₆₇₀₋₆₈₇ antibody) on native VSOAC currents in PSMCs. Membrane currents were obtained by applying 100-ms step pulses to ± 80 mV from a holding potential of -40 mV every 30 s. In Fig. 3, A and B, the time courses of change in the amplitudes of membrane currents measured at ± 80 mV are shown, and original current traces obtained at the time points indicated by small letters were depicted in the insets. In a cell dialyzed with 10 μ g/ml C₆₇₀₋₆₈₇ antibody for over 10 min, basal membrane currents gradually declined in isotonic bath solution. Subsequent hypotonic cell swelling failed to induce any increase of the current amplitude (Fig. 3A). To know whether the observed inhibitory effects of C₆₇₀₋₆₈₇ antibody on VSOAC were due to specific binding, similar experiments were repeated with the antigen-preabsorbed C₆₇₀₋₆₈₇ antibody. Figure 3B shows a representative experiment. In contrast to the effects observed with the C₆₇₀₋₆₈₇ antibody alone, dialysis with 10 μ g/ml antigen-preabsorbed C₆₇₀₋₆₈₇ antibody for over 10 min did not prevent activation of VSOAC on hypotonic cell swelling. Sub-

sequent exposure of the cell to hypertonic bath solution totally reversed the swelling-induced VSOAC currents. Figure 3C summarizes the current densities obtained in control cells and in cells dialyzed with either C₆₇₀₋₆₈₇ antibody or antigen-preabsorbed C₆₇₀₋₆₈₇ antibody. In five cells dialyzed with control pipette solution, hypotonic cell swelling significantly increased VSOAC current density at $+80$ mV (7.76 ± 0.70 pA/pF in hypotonic solution vs. 2.71 ± 0.71 pA/pF in isotonic solution). In eight cells dialyzed with 10 μ g/ml C₆₇₀₋₆₈₇ antibody, current density measured at $+80$ mV under hypotonic condition (2.06 ± 0.28 pA/pF) was not significantly different from that in isotonic solution (2.19 ± 0.34 pA/pF). However, in cells dialyzed with antigen-preabsorbed C₆₇₀₋₆₈₇ antibody, hypotonic cell swelling caused a significant increase in currents density at $+80$ mV (6.32 ± 0.75 pA/pF in hypotonic solution vs. 1.87 ± 0.10 pA/pF in isotonic solution, $n = 8$), which is not significantly different from the swelling-induced VSOAC currents in control group.

Similar to C₆₇₀₋₆₈₇ antibody, another anti-CLC-3 antibody raised against an amino-terminal epitope of CLC-3 (A₁₋₁₄ antibody) also demonstrated blocking effects on VSOACs in PSMCs. Figure 4A illustrates the time course of change in VSOAC current amplitudes recorded at ± 80 mV in two cells dialyzed with either 2.5 μ g/ml A₁₋₁₄ antibody or antigen-preabsorbed antibody. Intracellular dialysis with the A₁₋₁₄ antibody for

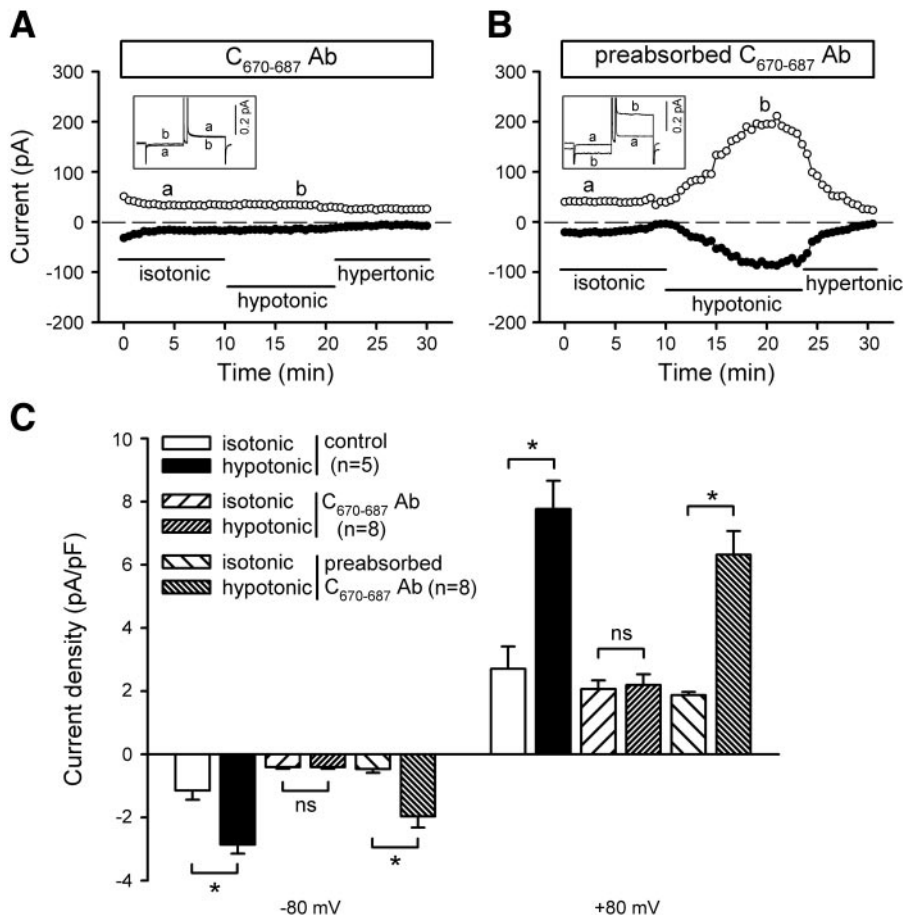
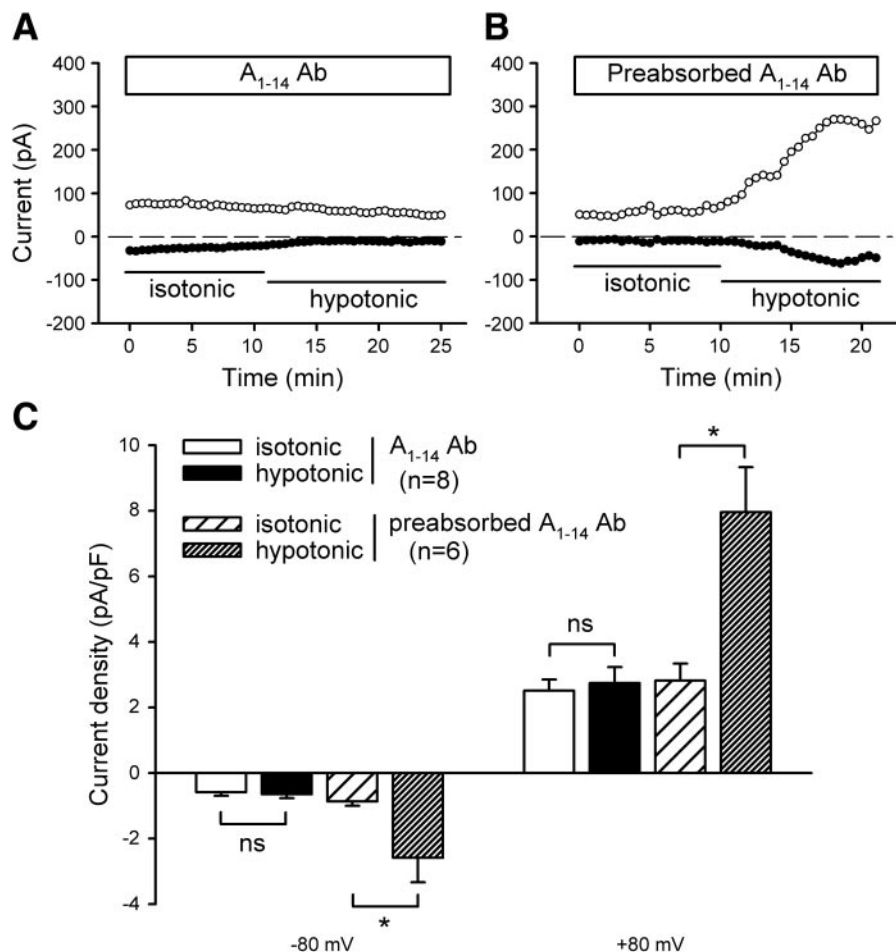


Fig. 3. Inhibition of native volume-sensitive organic osmolyte and anion channels (VSOACs) in canine PSMCs by anti-CLC-3 C₆₇₀₋₆₈₇ antibody intracellular dialysis. Membrane currents were induced by repetitive 100-ms voltage steps to ± 80 mV from a holding potential of -40 mV every 30 s. A and B: time courses of change in current amplitude measured at both -80 mV (filled circles) and $+80$ mV (open circles) in two representative cells intracellularly dialyzed with either 10 μ g/ml CLC-3 C₆₇₀₋₆₈₇ antibody (A) or the antigen-preabsorbed C₆₇₀₋₆₈₇ antibody (B). Horizontal solid lines underneath the current traces indicate different bath solutions. Original current recordings obtained at the time points indicated by lowercase letters were shown in the corresponding insets. C: summarized data comparing membrane current densities under isotonic and hypotonic conditions obtained from control cells as well as cells dialyzed with either 10 μ g/ml CLC-3 C₆₇₀₋₆₈₇ antibody or the antigen-preabsorbed C₆₇₀₋₆₈₇ antibody. Membrane potentials at which the currents were measured are indicated at the bottom. * $P < 0.05$ vs. respective isotonic conditions. ns, Not significant.

Fig. 4. Anti-CLC-3 A_{1-14} antibody intracellular dialysis inhibits native VSOACs in canine PSMCs. Membrane currents were induced by repetitive 100-ms voltage steps to ± 80 mV from a holding potential of -40 mV every 30 s. *A* and *B*: time courses of change in current amplitudes recorded at -80 mV (filled circles) and $+80$ mV (open circles) in two representative cells intracellularly dialyzed with either 2.5 $\mu\text{g/ml}$ CLC-3 A_{1-14} antibody (*A*) or the antigen-preabsorbed A_{1-14} antibody (*B*). Horizontal solid lines underneath the current traces indicate different bath solutions. *C*: summarized data comparing membrane current densities under isotonic and hypotonic conditions obtained from the cells dialyzed with either 10 $\mu\text{g/ml}$ CLC-3 A_{1-14} antibody or the antigen-preabsorbed A_{1-14} antibody. Membrane potentials at which the currents were measured are indicated at the bottom. * $P < 0.05$ vs. respective isotonic conditions.



over 10 min prevented swelling-induced activation of VSOACs (Fig. 4A). Preabsorption of the A_{1-14} antibody with antigen reversed the inhibitory effects of the antibody on VSOACs (Fig. 4B). Summarized current densities are shown in Fig. 4C. In eight cells dialyzed with 2.5 $\mu\text{g/ml}$ A_{1-14} antibody, current density measured in hypotonic solution did not differ significantly from that under isotonic conditions. In cells dialyzed with antigen-preabsorbed A_{1-14} antibody, swelling-induced VSOAC currents amounted to 7.959 ± 1.372 pA/pF at $+80$ mV, which was significantly larger than the basal currents under isotonic conditions (1.869 ± 0.103 pA/pF at $+80$ mV) but not significantly different from the swelling-induced VSOAC currents in control cells.

Additional experiments were performed in which the effects of the new antibody raised against an extracellular epitope ($\text{Ex}_{133-146}$) of CLC-3 were examined in PSMCs (data not shown). After the swelling-induced VSOAC currents reached steady state, application of 1.6 $\mu\text{g/ml}$ $\text{Ex}_{133-148}$ antibody to the bath solution did not cause any noticeable change of VSOAC current amplitude, and exposure of the cell to hypertonic solution completely reversed the swelling-induced VSOAC currents. At $+80$ mV, the membrane current density was 1.841 ± 0.716 pA/pF under isotonic conditions and significantly increased to 5.418 ± 0.722 pA/pF in hypotonic solutions. After treatment with $\text{Ex}_{133-148}$ anti-

body, the current density amounted to 5.984 ± 1.104 pA/pF, which was not significantly different from that under the hypotonic condition ($n = 4$). Similar results were obtained when cells were first incubated with 1.6 $\mu\text{g/ml}$ $\text{Ex}_{133-148}$ antibody in isotonic solution for 30 min and then challenged with hypotonic solution (data not shown).

Blockade of VSOACs in guinea pig atrial myocytes by anti-CLC-3 $C_{670-687}$ antibody dialysis. Another type of tissue where a causal relation between native CLC-3 expression and VSOAC has been suggested is guinea pig cardiac muscle. Therefore, we examined whether the inhibitory effects of $C_{670-687}$ antibody on VSOACs in PSMC could also be demonstrated in guinea pig atrial myocytes. In Fig. 5, *A* and *B*, the time course of change in the amplitude of membrane currents measured at ± 80 mV in cells dialyzed with either 10 $\mu\text{g/ml}$ $C_{670-687}$ antibody or antigen-preabsorbed $C_{670-687}$ antibody is illustrated. The current-voltage relation measured under isotonic and hypotonic conditions at test potentials ranging from -100 to $+120$ mV in 20-mV increments from a holding potential of -40 mV are depicted in the corresponding insets in Fig. 5. In a cell dialyzed with $C_{670-687}$ antibody for 15 min, hypotonic cell swelling failed to induce any increase in membrane currents (Fig. 5A). This inhibitory effect of the antibody on VSOACs could be prevented in a cell dialyzed with

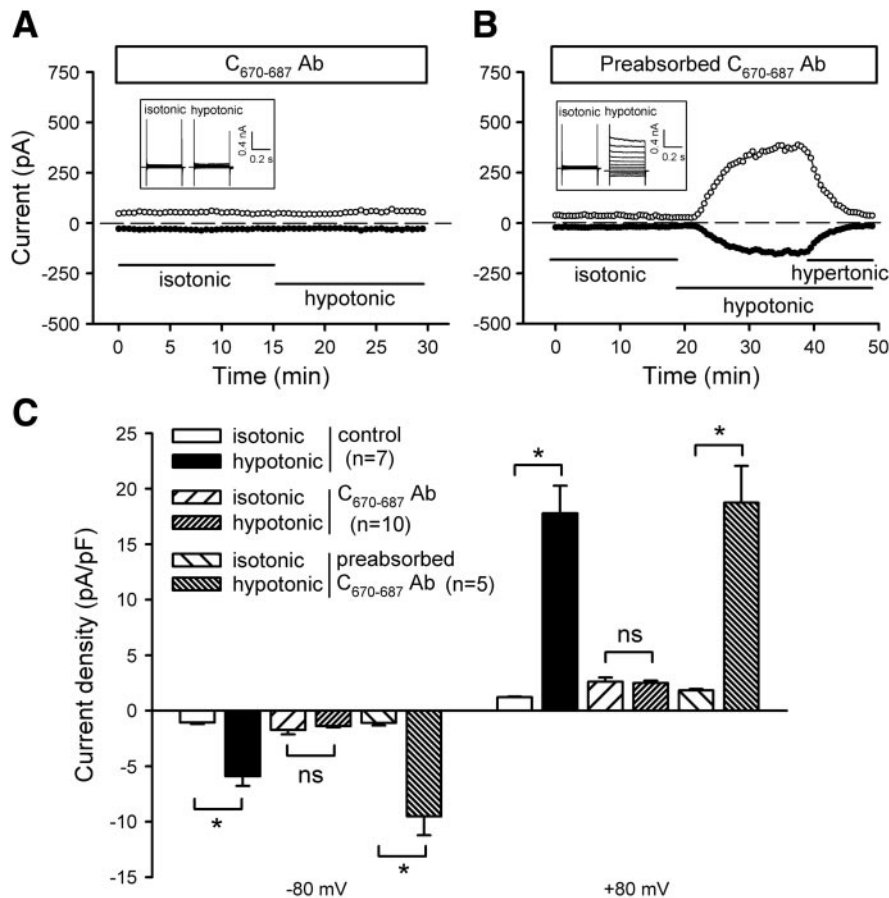


Fig. 5. Inhibition of native VSOACs in guinea pig atrial myocytes by anti-CLC-3 $C_{670-687}$ antibody intracellular dialysis. Membrane currents were induced by repetitive 100-ms voltage steps to ± 80 mV from a holding potential of -40 mV every 30 s. **A** and **B**: time courses of change in current amplitudes at both -80 mV (filled circles) and $+80$ mV (open circles) recorded from two representative cells intracellularly dialyzed with either $10 \mu\text{g/ml}$ CLC-3 $C_{670-687}$ antibody (**A**) or the antigen-preabsorbed $C_{670-687}$ antibody (**B**). Horizontal solid lines underneath the current traces indicate different bath solutions. Original membrane current traces depicted in the insets were recorded under 400-ms voltage steps ranging from -100 to $+120$ mV in 20-mV increments from a holding potential of -40 mV every 2 s. **C**: summarized data comparing membrane current densities under isotonic and hypotonic conditions obtained from control cells as well as cells dialyzed with either $10 \mu\text{g/ml}$ CLC-3 $C_{670-687}$ antibody or the antigen-preabsorbed $C_{670-687}$ antibody. Membrane potentials at which the currents were measured are indicated at the bottom. $*P < 0.05$ vs. respective isotonic conditions.

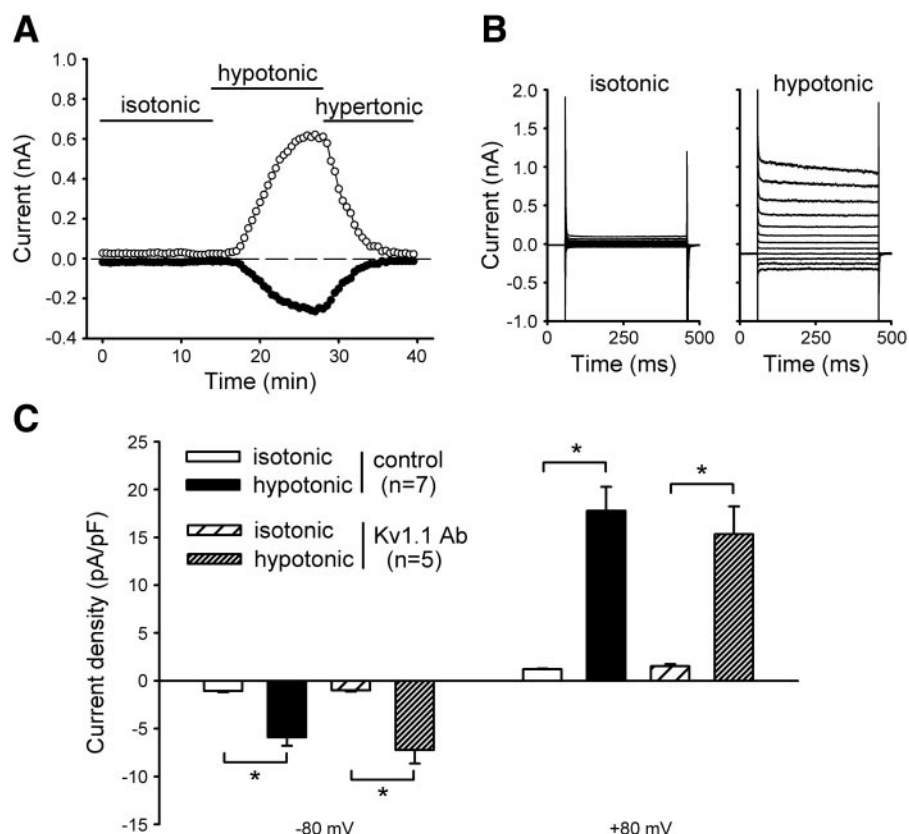
antigen-preabsorbed $C_{670-687}$ antibody (Fig. 5B). As can be seen in the inset of Fig. 5B, the swelling-induced currents displayed outward rectification and inactivation at positive membrane potentials. Figure 5C summarizes the data obtained from control cells and cells dialyzed with either $10 \mu\text{g/ml}$ $C_{670-687}$ antibody or antigen-preabsorbed $C_{670-687}$ antibody. In control cells, hypotonic cell swelling caused a significant increase in the current amplitudes measured at ± 80 mV. Under isotonic and hypotonic conditions, the mean current densities at $+80$ mV obtained from seven cells amounted to 1.21 ± 0.06 and 17.8 ± 2.50 pA/pF, respectively. Similar increase of VSOAC current density on hypotonic cell swelling was observed in cells dialyzed with antigen-preabsorbed $C_{670-687}$ antibody. At $+80$ mV, averaged membrane current densities were 1.83 ± 0.13 and 18.7 ± 3.32 pA/pF under isotonic and hypotonic conditions, respectively. In contrast, in cells dialyzed with $10 \mu\text{g/ml}$ $C_{670-687}$ antibody, there was no significant change in the mean current densities measured at $+80$ mV in the hypotonic solution (2.60 ± 0.39 pA/pF) compared with the isotonic condition (2.50 ± 0.20 pA/pF).

Lack of effect of anti-Kv1.1 antibody on VSOACs in guinea pig atrial myocytes. To further verify the specificity of the inhibitory effects of the new anti-CLC-3 antibodies on VSOACs, we conducted experiments using an antibody (rabbit) against the Kv1.1 potassium

channel (Kv1.1 antibody). As shown in Fig. 6A, intracellular dialysis with $10 \mu\text{g/ml}$ Kv1.1 antibody in a guinea pig atrial myocyte for over 15 min did not prevent the increase in the amplitude of VSOAC current induced by hypotonic solution. Figure 6B depicts the respective original current traces recorded in isotonic and hypotonic solutions elicited by voltage-clamp steps ranging from -100 to $+120$ mV. The average current densities at ± 80 mV are compared in Fig. 6C. In cells dialyzed with Kv1.1 antibody, hypotonic cell swelling induced large increases in VSOAC current densities (15.3 ± 2.91 in hypotonic solution vs. 1.53 ± 0.22 pA/pF in isotonic solution at $+80$ mV), which were not significantly different from the swelling-induced VSOAC currents in control cells.

Anti-CLC-3 $C_{670-687}$ antibody dialysis does not affect swelling-induced augmentation of I_{Ks} . It has been shown previously that the activity of I_{Ks} is enhanced by hypotonic cell swelling (24). As an additional control experiment to test the specificity of $C_{670-687}$ antibody, we examined whether intracellular dialysis of $C_{670-687}$ antibody would affect the modulation of I_{Ks} by cell swelling. Figure 7, **A** and **B**, illustrates the representative currents obtained from guinea pig ventricular myocytes dialyzed with either control pipette solution or with $10 \mu\text{g/ml}$ $C_{670-687}$ antibody. Membrane currents were elicited by 2-s depolarizing pulses to potentials ranging from -20 to $+80$ mV from a holding

Fig. 6. Lack of effect of intracellular dialysis of anti-Kv1.1 antibody on native VSOACs in guinea pig atrial myocytes. Membrane currents were induced by repetitive 100-ms voltage steps to ± 80 mV from a holding potential of -40 mV every 30 s. **A**: representative traces showing time course of change in the current amplitudes recorded at -80 mV (filled circles) and $+80$ mV (open circles) in a cell intracellularly dialyzed with $10 \mu\text{g/ml}$ anti-Kv1.1 antibody. Horizontal solid lines above the traces indicate different bath solutions. **B**: original membrane current traces from the same cell recorded at potentials ranging from -100 to $+120$ mV in 20-mV increments from a holding potential of -40 mV every 2 s. **C**: summarized data comparing membrane current densities under isotonic and hypotonic conditions obtained from control cells as well as cells dialyzed with $10 \mu\text{g/ml}$ anti-Kv1.1 antibody. Membrane potentials at which the currents were measured are indicated at the bottom. $*P < 0.05$ vs. respective isotonic conditions.



potential of -40 mV every 5 s. The initial time-independent currents elicited at the beginning of voltage steps to potentials greater than and equal to $+40$ mV should mainly reflect VSOAC currents, because the rapid component of the delayed rectifying potassium currents (I_{Kr}) is almost completely inactivated at these positive potentials and I_{Ks} is not yet activated (25). In a cell dialyzed with control pipette solution (Fig. 7A), hypotonic cell swelling not only caused an increase in the amplitude of the initial time-independent current but also remarkably augmented the late time-dependent I_{Ks} amplitudes measured at the end of the test pulses. The net swelling-sensitive currents obtained by subtracting the current traces under isotonic conditions from that under hypotonic conditions are shown in Fig. 7A, right. Currents increased by cell swelling included an initial time-independent component (VSOACs) as well as a slow time-dependent component (I_{Ks}). In contrast, when a cell was dialyzed with $C_{670-687}$ antibody (Fig. 7B), hypotonic cell swelling only increased the slow time-dependent I_{Ks} but did not activate the initial time-independent current due to VSOACs. Figure 7C compares the mean normalized current amplitudes obtained at $+60$ mV under isotonic and hypotonic conditions in control cells as well as in cells dialyzed with $C_{670-687}$ antibody. In control cells, the amplitudes of both the initial time-independent currents and the slow time-dependent currents increased significantly on hypotonic cell swelling. In cells dialyzed with $C_{670-687}$ antibody, hypotonic solution caused a similar increase in the slow time-dependent I_{Ks}

as in control cells; however, activation of the initial time-independent VSOAC currents by hypotonic cell swelling was not observed.

DISCUSSION

Despite several lines of evidence supporting a role of ClC-3 as a molecular candidate for VSOACs in some types of mammalian cells (7, 8, 14, 33), a series of recent studies have challenged the validity of the ClC-3 hypothesis (16, 18, 27, 34). However, these studies are not without limitations of their own, with many results inconsistent between different laboratories or even within the same laboratory (15, 18, 34). For example, expression of rat short ClC-3 isoform (homologous to gpClC-3) in Chinese hamster ovary (CHO)-K1 cells has been reported to yield Cl^- currents with biophysical properties different from native VSOACs (18, 26), although no evidence was provided to clearly distinguish Cl^- currents due to ClC-3 isoform expression from endogenous Cl^- currents known to be present in CHO-K1 cells. The anion selectivity for the short ClC-3 isoform has been reported by this same laboratory to be both $\text{I}^- > \text{Cl}^-$ and $\text{Cl}^- > \text{I}^-$. Expression of the human long isoform of ClC-3 into tsA cells was reported to yield Cl^- currents regulated by calcium/calmodulin-dependent protein kinase II (CaMKII) (16), whereas another study failed to even detect functional Cl^- channels following expression of the human long ClC-3 isoform (34).

Native VSOACs in hepatocytes and pancreatic acinar cells have been reported to be unaltered in mice with

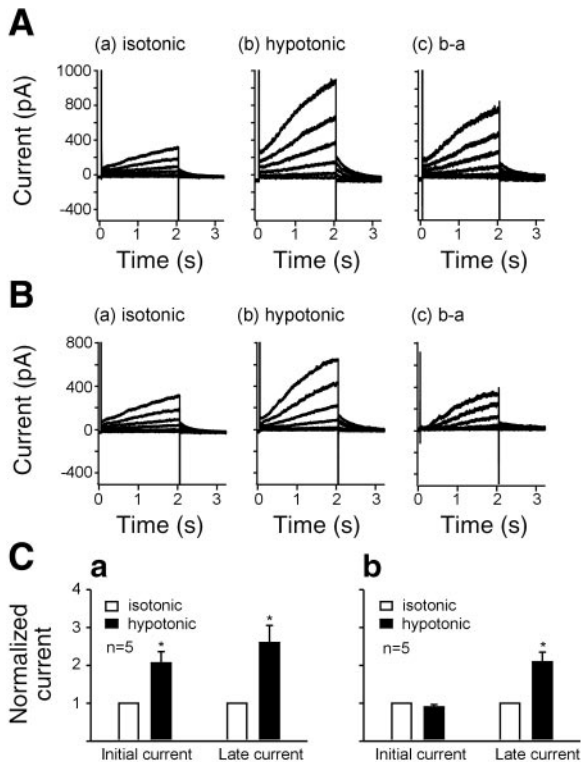


Fig. 7. Lack of effect of intracellular dialysis of anti-CLC-3 $C_{670-687}$ antibody on cell swelling modulation of delayed rectifier potassium current (I_{Ks}) in guinea pig ventricular myocytes. Membrane currents were elicited by 2-s depolarizing pulses to potentials ranging from -20 to $+80$ mV in 20-mV increments from a holding potential of -40 mV every 5 s. *A* and *B*: representative original current traces obtained from cells intracellularly dialyzed with either control pipette solution (*A*) or 10 μ g/ml $C_{670-687}$ antibody (*B*). Net swelling-sensitive currents displayed in *C*, *a* and *b*, were obtained by subtracting the current traces under isotonic condition from that under hypotonic condition. *C*: summarized averaged data showing normalized current amplitudes obtained at $+60$ mV under isotonic and hypotonic conditions in control cells (*a*) as well as in cells dialyzed with $C_{670-687}$ antibody (*b*). The initial time-independent current was measured after 5 ms from the beginning of each test pulse, whereas late, time-dependent outward currents were measured at the end of each test pulse. The currents obtained under hypotonic conditions were normalized to their respective currents measured under isotonic solutions in *C*. * $P < 0.05$ vs. respective values under isotonic conditions.

disrupted CLC-3 ($Clcn^{-/-}$ knockout) (27). This observation has been used as evidence against any role of CLC-3 as a volume-regulated Cl^{-} channel. However, it is well established that the phenotypic changes associated with conventional gene knockout models may only be observed in tissues, where the gene plays an irreplaceable functional role. Indeed, the apparent unaltered VSOACs observed in hepatocytes and pancreatic acinar cells of $Clcn^{-/-}$ mice might be due to the expression of different subtypes of VSOACs in these cells or due to an upregulation of a different VSOAC subtype in response to loss of CLC-3. The possibility of endogenous expression of different subtypes of VSOACs is suggested by the wide range of properties of native VSOACs reported in different cell types (21, 23, 28) and by the demonstrated lack of effect of CLC-3 antisense on a small subpopulation of native VSOACs in bovine epithelial cells (33). This possibility

needs to be tested more rigorously by carefully comparing the properties of native VSOACs in a wider range of different cell types from $Clcn^{-/-}$ knockout mice. There is precedence for the possible existence of different VSOAC subtypes in vascular smooth muscle cells. VSOACs in the rabbit portal vein are stimulated by activation of protein kinase C (10), whereas VSOACs in canine pulmonary artery are inhibited by activation of protein kinase C (36), and preliminary data suggest that VSOACs in the portal vein may also be insensitive to inhibition by anti-CLC-3 antibodies as well (32). These differences suggest that different proteins may be responsible for native VSOACs in various cell types with distinct mechanisms of channel regulation by cell volume.

An advantage of using antibodies as acute functional probes for CLC-3 is the lack of activation of compensatory mechanisms that may be associated with conventional antisense or gene manipulation techniques. In a previous report, we demonstrated that intracellular dialysis with a commercially available anti-CLC-3 carboxy terminus (Alm $C_{592-661}$) antibody abolished VSOACs in several cell types, including cardiac and vascular smooth muscle cells (8). However, a major problem with the Alomone anti-CLC-3 antibody is its nonspecific reactivity with proteins other than CLC-3 (27, 34). In the present study, we further evaluated the relation between endogenous CLC-3 and native VSOACs by examining the functional effects of three new anti-CLC-3 antibodies on native VSOACs in canine PSMCs and guinea pig cardiac myocytes.

The affinity-purified forms of these three new antibodies produced a common prominent immunoreactive band with an apparent molecular mass of 90–92 kDa in the guinea pig heart and PSMCs (Fig. 2). A similar molecular mass immunoreactive band was observed using the $C_{670-687}$ antibody in the brains from homozygous $Clcn3^{+/+}$ mice, but not from homozygous $Clcn3^{-/-}$ mice, suggesting this immunoreactive 90- to 92-kDa signal indeed represents CLC-3 protein. Further support comes from the recent demonstration that CLC-3 antisense oligonucleotides or antisense cRNA significantly reduce the density of a similar molecular mass band using both Alm $C_{592-661}$ antibody and $C_{670-687}$ antibody in immunoblots in HeLa cells and *Xenopus* oocytes, respectively (14). Improved specificity of the new antibodies (A_{1-14} , $Ex_{133-148}$, and $C_{670-687}$) for CLC-3 is suggested by the relative absence of additional immunoreactive bands in Western blots in cardiac tissue and PSMCs compared with the commercial Alm $C_{592-661}$ antibody, which exhibited numerous immunoreactive bands with a wide range of molecular masses (Fig. 2).

Similar to dialysis with the commercially available anti-CLC-3 Alm $C_{592-661}$ antibody (8), intracellular dialysis with either $C_{670-687}$ antibody or A_{1-14} antibody prevented activation of native VSOACs by hypotonic cell swelling in canine PSMCs as well as in guinea pig cardiac myocytes. The lack of inhibition of VSOACs by the corresponding antigen-preabsorbed antibodies suggests that the inhibitory effects of these antibodies did not result from nonspecific binding. The blockade of VSOACs by the anti-CLC-3 antibodies could be due to

direct effects of the antibodies on the channel protein itself or caused by interference of the antibodies with cellular mechanisms mediating the swelling-induced activation of VSOACs. However, the fact that three antibodies (C₆₇₀₋₆₈₇, A₁₋₁₄, and Alm C₅₉₂₋₆₆₁) targeting different distinct epitopes of CLC-3 exhibit the same blocking effects on native VSOACs is strong evidence that a direct interaction of the antibodies with CLC-3 protein underlies the observed inhibitory effects of the antibodies on native VSOACs. To exclude the possibility that intracellular dialysis with antibodies may non-specifically interfere with the function of VSOACs and lead to inhibition of the swelling-induced Cl⁻ currents, we determined the effects on VSOACs of a specific antibody against the Kv1.1 potassium channels. In contrast to the anti-CLC-3 antibodies, intracellular dialysis with the Kv1.1 antibody did not cause any inhibition of VSOACs in guinea pig atrial myocytes. This result further suggests that blockade of VSOACs by the anti-CLC-3 antibodies was not caused by nonspecific binding but due to unique reactivity of the anti-CLC-3 antibodies with VSOACs.

Additional evidence for the specificity of the inhibitory effect of the anti-CLC-3 antibodies on VSOACs was obtained from experiments where the effects of C₆₇₀₋₆₈₇ antibody on the swelling-induced activation of VSOACs and I_{Ks} were directly compared in the same guinea pig myocytes. In cardiac myocytes, cell swelling not only elicits activation of VSOACs but also alters the activity of many other types of ion channels (30). It has been shown previously that the activity of I_{Ks} in cardiac ventricular myocytes is significantly enhanced by hypotonic cell swelling (24). We confirmed this observation in the present study. Furthermore, our experiments showed that intracellular dialysis with anti-CLC-3 C₆₇₀₋₆₈₇ antibody did not prevent augmentation of I_{Ks} by hypotonic cell swelling, although swelling-induced activation of VSOACs was abolished in the same cells. This result clearly demonstrates that C₆₇₀₋₆₈₇ antibody had no functional cross-reactivity with the I_{Ks} channel nor did it interfere with the signaling pathways mediating swelling-induced augmentation of I_{Ks} .

Whereas our data support the role of endogenous CLC-3 as a protein responsible for native VSOACs in guinea pig cardiac myocytes and canine PASM, Huang et al. (16) reported that expression of hCLC-3 into tsA epithelial cells resulted in Cl⁻-selective currents regulated by CaMKII, with little change in the density of endogenous VSOACs. The different current phenotype observed in response to expression of hCLC-3 in this study might be attributable to possible functional differences in properties of the long isoform of hCLC-3 expressed in these experiments compared with the VSOAC role attributed to expression of the short isoform of CLC-3 (7). It is known that the long isoform of CLC-3 differs significantly from the short CLC-3 isoform by 58 extra amino acids in the amino-terminal region, which may account for possible differences in functional phenotype of the two isoforms. Interestingly, Huang et al. (16) demonstrated that di-

alysis with a polyclonal anti-CLC-3 antibody raised against an NH₂-terminal epitope similar (but not identical) to the A₁₋₁₄ antibody used in the present study failed to inhibit CaMKII-activated Cl⁻ currents or native VSOACs in these cells. Another antibody targeting a carboxy-terminal epitope, in contrast, was found to be effective in inhibiting CaMKII-activated Cl⁻ currents without affecting native VSOACs.

Although the present study does not completely exclude the possibility that endogenous CLC-3 in guinea pig cardiac myocytes and canine PASMcs may serve as a regulator of native VSOACs, the following additional results strongly support CLC-3 as directly responsible for some types of native VSOACs: 1) heterologous expression of gpCLC-3 gives rise to large outwardly rectifying volume-sensitive Cl⁻ currents with properties very similar to the native VSOACs, 2) site-directed mutations change the anion selectivity and rectification of the expressed gpCLC-3 channel (7) and its regulation by protein kinase C and cell volume (5), and 3) CLC-3 antisense oligonucleotide treatment dramatically reduce native VSOAC current densities and regulatory volume decreases in Hela cells and *Xenopus* oocytes (14).

At present, the exact mechanisms by which binding of the anti-CLC-3 antibodies to CLC-3 channel proteins lead to inhibition of VSOACs is unclear. The recently resolved crystal structure of the bacterial CLC channels (9) shows that both the amino and carboxy terminal of CLC are in the vicinity of the channel pore. Thus it seems likely that binding of the anti-CLC-3 antibodies to either the amino terminus or the carboxy terminus of CLC-3 may block the channel pore directly. Alternatively, anti-CLC-3 antibodies may interfere with the gating mechanisms of CLC-3. The ineffectiveness of the extracellular anti-CLC-3 antibody to block native VSOACs is consistent with the CLC crystal structure showing that the extracellular location of residues 133-148 are far from the putative pore region of the channel. Clearly, further experiments are required to further elucidate the exact mechanism of block of native VSOACs by anti-CLC-3 antibodies demonstrated here.

In summary, the finding that native VSOACs in guinea pig cardiac myocytes and canine PASMcs are functionally inhibited by intracellular dialysis of three different anti-CLC-3 antibodies (C₆₇₀₋₆₈₇, A₁₋₁₄, and Alm C₅₉₂₋₆₆₁) raised against distinct amino acid epitopes is strong evidence supporting a major role of endogenous CLC-3 as the protein responsible for native VSOACs in these cell types. The functional effects of the two novel antibodies (C₆₇₀₋₆₈₇ and A₁₋₁₄) described here should make them useful tools in future studies to unequivocally establish the identity of transgenically expressed CLC-3 Cl⁻ currents in various heterologous expression systems.

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DISCLOSURES

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REFERENCES

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, and Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389–3402, 1997.
- Britton FC, Hatton WJ, Rossow CF, Duan D, Hume JR, and Horowitz B. Molecular distribution of volume regulated chloride channels (ClC-2, ClC-3) in cardiac tissues. *Am J Physiol Heart Circ Physiol* 279: H2225–H2233, 2000.
- Clapham DE. The list of potential volume-sensitive chloride currents continues to swell. *J Gen Physiol* 111: 623–624, 1998.
- Dickerson LW, Bonthius DJ, Schutte BC, Yang B, Barna TJ, Carter MC, Nehrke K, Williamson RA, and Lamb FS. Altered GABAergic function accompanies hippocampal degeneration in mice lacking ClC-3 voltage-gated chloride channels. *Brain Res* 958: 227–250, 2002.
- Duan D, Cowley S, Horowitz B, and Hume JR. A serine residue in ClC-3 links phosphorylation-dephosphorylation to chloride channel regulation by cell volume. *J Gen Physiol* 113: 57–70, 1999.
- Duan D, Hume JR, and Nattel S. Evidence that outwardly rectifying Cl⁻ channels underlie volume-regulated Cl⁻ currents in heart. *Circ Res* 80: 103–113, 1997.
- Duan D, Winter C, Cowley S, Hume JR, and Horowitz B. Molecular identification of a volume-regulated chloride channel. *Nature* 390: 417–421, 1997.
- Duan D, Zhong J, Hermoso M, Satterwhite CM, Rossow CF, Hatton WJ, Yamboliev I, Horowitz B, and Hume JR. Functional inhibition of native volume-sensitive outwardly rectifying anion channels in muscle cells and *Xenopus* oocytes by anti-ClC-3 antibody. *J Physiol* 531: 437–444, 2001.
- Dutzler R, Campbell EB, Cadene M, Chait BT, and Mackinnon R. X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. *Nature* 415: 287–294, 2002.
- Ellershaw DC, Greenwood IA, and Large WA. Modulation of volume-sensitive chloride current by noradrenergic in rabbit portal vein myocytes. *J Physiol* 542: 537–547, 2002.
- Gentzsch M, Cui L, Mengos A, Chang XB, Chen JH, and Riordan JR. The PDZ-binding chloride channel ClC-3B localizes to the golgi and associates with cystic fibrosis transmembrane conductance regulator-interacting PDZ proteins. *J Biol Chem* 278: 6440–6449, 2003.
- George AL Jr, Bianchi L, Link EM, and Vanoye CG. From stones to bones: the biology of ClC chloride channels. *Curr Biol* 11: R620–R628, 2001.
- Hamill OP, Marty A, Neher B, and Sigworth FJ. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 391: 85–100, 1981.
- Hermoso M, Satterwhite CM, Andrade YN, Hidalgo J, Wilson SM, Horowitz B, and Hume JR. ClC-3 is a fundamental molecular component of volume-sensitive outwardly rectifying Cl⁻ channels and volume regulation in HeLa cells and *Xenopus laevis* oocytes. *J Biol Chem* 277: 40066–40074, 2002.
- Higgins CF, Weylandt KH, Nastrucci C, Sardini A, Linton K, Diaz M, and Valverde MA. Cell swelling-activated chloride channels and their regulation by P-glycoprotein. In: *Chloride Channels*, edited by Kozlowski R. Oxford, UK: Isis Medical Media, 1999, p. 35–46.
- Huang P, Liu J, Di A, Robinson NC, Musch MW, Kaetzel MA, and Nelson DJ. Regulation of human ClC-3 channels by multifunctional Ca²⁺/calmodulin-dependent protein kinase. *J Biol Chem* 276: 20093–20100, 2001.
- Jentsch TJ, Stein V, Weinreich F, and Zdebik AA. Molecular structure and physiological function of chloride channels. *Physiol Rev* 82: 503–568, 2002.
- Li X, Shimada K, Showalter LA, and Weinman SA. Biophysical properties of ClC-3 differentiate it from swelling-activated chloride channels in Chinese hamster ovary-K1 cells. *J Biol Chem* 275: 35994–35998, 2000.
- Mindell JA and Maduke M. ClC chloride channels (Reviews). *Genome Biol* 2: 3003.1–3003.6, 2001.
- Nastrucci C, Diaz M, Weylandt KH, Sardini A, and Higgins CF. Expression of human ClC-3 in NIH3T3 fibroblasts is associated with volume-activated, outwardly rectifying chloride currents. *J Physiol* 517P: 74P–75P, 1999.
- Nilius B, Eggermont J, Voets T, Buyse G, Manolopoulos V, and Droogmans G. Properties of volume-regulated anion channels in mammalian cells. *Prog Biophys Mol Biol* 68: 69–119, 1997.
- Ogura T, Furukawa T, Toyozaki T, Yamada K, Zheng YJ, Katayama Y, Nakaya H, and Inagaki N. ClC-3B, a novel ClC-3 splicing variant that interacts with EBP50 and facilitates expression of CFTR-regulated ORCC. *FASEB J* 16: 863–865, 2002.
- Okada Y. Volume expansion-sensing outward-rectifier Cl⁻ channel: fresh start to the molecular identity and volume sensor. *Am J Physiol Cell Physiol* 273: C755–C789, 1997.
- Rees SA, Vandenberg JI, Wright AR, Yoshida A, and Powell T. Cell swelling has differential effects on the rapid and slow components of delayed rectifier potassium current in guinea pig cardiac myocytes. *J Gen Physiol* 106: 1151–1170, 1995.
- Sanguinetti MC and Jurkiewicz NK. Two components of cardiac delayed rectifier K⁺ current. Differential sensitivity to block by class III antiarrhythmic agents. *J Gen Physiol* 96: 195–215, 1990.
- Shimada K, Li X, Xu G, Nowak DE, Showalter LA, and Weinman SA. Expression and canalicular localization of two isoforms of the ClC-3 chloride channel from rat hepatocytes. *Am J Physiol Gastrointest Liver Physiol* 279: G268–G276, 2000.
- Stobrawa SM, Breiderhoff T, Takamori S, Engel D, Schweizer M, Zdebik AA, Bosl MA, Ruether K, Jahn H, Draguhn A, Jahn R, and Jentsch TJ. Disruption of ClC-3, a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. *Neuron* 29: 185–196, 2001.
- Strange K, Emma F, and Jackson PS. Cellular and molecular physiology of volume-sensitive anion channels. *Am J Physiol Cell Physiol* 270: C711–C730, 1996.
- Valverde MA. ClC channels: leaving the dark ages on the verge of a new millennium. *Curr Opin Cell Biol* 11: 509–516, 1999.
- Vandenberg JI, Rees SA, Wright AR, and Powell T. Cell swelling and ion transport pathways in cardiac myocytes. *Cardiovasc Res* 32: 85–97, 1996.
- Waldegger S and Jentsch TJ. From tonus to tonicity: physiology of ClC chloride channels. *J Am Soc Nephrol* 11: 1331–1339, 2000.
- Wang GX, Hatton WJ, Yamboliev I, Zhong J, and Hume JR. Volume-sensitive outwardly rectifying anion channel (VSOAC) subtypes in smooth muscle cells revealed by sensitivity to specific inhibitory ClC-3 antibodies (Abstract). *Biophys J* 82: 13a, 2002.
- Wang L, Chen L, and Jacob TJ. The role of ClC-3 in volume-activated chloride currents and volume regulation in bovine epithelial cells demonstrated by antisense inhibition. *J Physiol* 524.1: 63–75, 2000.
- Weylandt KH, Valverde MA, Nobles M, Raguz S, Amey JS, Diaz M, Nastrucci C, Higgins CF, and Sardini A. Human ClC-3 is not the swelling-activated chloride channel involved in cell volume regulation. *J Biol Chem* 276: 17461–17467, 2001.
- Yamazaki J, Duan D, Janiak R, Kuenzli K, Horowitz B, and Hume JR. Functional and molecular expression of volume-regulated chloride channels in canine vascular smooth muscle cells. *J Physiol* 507: 729–736, 1998.
- Zhong J, Wang GX, Hatton WJ, Yamboliev I, Walsh MP, and Hume JR. Regulation of volume-sensitive anion channels in pulmonary arterial smooth muscle cells by PKC. *Am J Physiol Cell Physiol* 283: C1627–C1636, 2002.