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

Ge-Xin Wang, William J. Hatton, Grace L. Wang, Juming Zhong, Ilia Yamboliev, Dayue Duan, and Joseph R. Hume

Center of Biomedical Research Excellence, Department of Pharmacology, University of Nevada School of Medicine Reno, Nevada 89557-0046

Submitted 18 March 2003; accepted in final form 12 June 2003

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 C592–661 antibody), although recent studies have raised questions related to the specificity of Alm C592–661 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 (C670–687 antibody) and amino terminus (A1–14 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 (EX133–148) 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 C670–687 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.

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 (IgpClC-3) (5); and 3) native VSOACs in cardiac and smooth muscle cells and Xenopus oocytes, as well as expressed IgpClC-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 CIC-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 CIC-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).

Address for reprint requests and other correspondence: J. R. Hume, Dept. of Pharmacology 318, Univ. of Nevada School of Medicine, Reno, NV 89557-0046 (E-mail: joeh@med.unr.edu).

http://www.ajpheart.org 0363-6135/03 $5.00 Copyright © 2003 the American Physiological Society
It has recently been suggested from studies of Cldn−/− mice that CIC-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 CIC-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-CIC-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-CIC-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 CIC-3 as the protein responsible for native VSOACs in smooth muscle and cardiac myocytes and 2) provide new experimental tools for future studies to distinguish Cl currents due to CIC-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.** CIC-3 belongs to the same branch of the CIC family as CIC-4 and CIC-5, which share ~80% identity (31). Attempts to produce CIC-3-specific antibodies therefore require peptide sequences to be designed from regions that show the least homology to CIC-4 and CIC-5. Peptide sequences selected for the production of CIC-3-specific antibodies were based on the following criteria: 1) specificity to CIC-3 isoforms, 2) lack of homology to the closely related CIC-4 and CIC-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 CIC-3 (short form), the resultant antibody referred to as A1–14 antibody; 2) KTWAELIIGQAEGPGS corresponding to residues 133–148 (16 amino acids), a region found in an extracellular portion between transmembrane domains D1 and D2 of mouse CIC-3 (short form), the resultant antibody referred to as EX133–148 antibody; and 3) GSSRVCFAQHTPSLPAES corresponding to residues 670–687 (18 amino acids), a region found at the carboxy terminus of mouse CIC-3 (short form), the resultant antibody referred to as C670–687 antibody.

The selected amino acid sequences were submitted to Research Genetics (Huntsville, AL) and assessed for suitability 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 CIC-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 CIC-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 A1–14, 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 CIC-3. Amino acid alignment of each peptide sequence with the closely related CIC-4 and CIC-5 proteins indicated that peptide A1–14 had 0 of 14 residues identical to CIC-4 and CIC-5; peptide EX133–148 had 6 of 16 and 5 of 16 residues identical to CIC-4 and CIC-5,
respective; peptide C_{670–687} 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 C_{670–687} 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 carboxyl-terminus residues 592–661 (70 amino acids), referred to as Alm C_{592–661} 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 carboxyl 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. Antiserum was 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 eliminate other ionic currents. Symmetrical chloride concentration of other ionic currents. Symmetrical chloride concentration of other ionic currents. Symmetrical chloride concentration of other ionic currents. Symmetrical chloride concentration of other ionic currents. Symmetrical chloride concentration of other ionic currents. Symmetrical chloride concentration of other ionic currents. Symmetrical chloride concentration of other ionic currents. Symmetrical chloride concentration of other ionic currents. Symmetrical chloride concentration of other ionic currents.

**Solutions and reagents.** Solutions and reagents.
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, 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 KCl, 5 Mg-ATP, 2 EGTA, 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 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. 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 for 10–14 days, and then dialyzed for 24 h against PBS (pH 7.4) at 4°C to achieve final concentrations. The osmolality of the pipette 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 $t_0$.

**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 PASMCs. A similar molecular mass immunoreactive band was also observed using the C670–687 antibody in the brains from homozgygous Clcn3 $^{-/-}$ mice but not from homozygous Clcn3 $^{+/+}$ mice (Fig. 2C), 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 commercial Alm C592–661 antibody in PASMCs (C670–687 antibody + AP).

**Fig. 2.** Western blot analysis of anti-ClC-3 antibodies in the guinea pig (GP, A) heart, canine pulmonary arterial smooth muscle cells (PASMCs, 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 PASMCs (B). A similar molecular mass immunoreactive band was observed using the C670–687 antibody (Ab) in the brain from homogous Clcn3 $^{-/-}$ mice but not from homoygous Clcn3 $^{+/+}$ mice (C), suggesting the immunoreactive 90- to 92-kDa signal is specific for ClC-3 protein. This ClC-3 immunoreactive signal was not observed in control blots using the secondary antibody alone in the GP heart or PASMCs or with the antigen-preabsorbed (AP) C670–687 antibody in PASMCs (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 PASMCs. 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 PASMCs. 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 μg/ml C670–687 antibody intracellular dialyz with the A1–14 antibody alone, dialysis with 10 μg/ml antigen-preabsorbed C670–687 antibody for over 10 min did not prevent activation of VSOAC on hypotonic cell swelling. Subsequent 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 μg/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 PASMCs. Figure 4A illustrates the time course of change in VSOAC current amplitudes recorded at ±80 mV in two cells dialyzed with either 2.5 μg/ml A1–14 antibody or antigen-preabsorbed antibody. Intracellular dialysis with the A1–14 antibody for
over 10 min prevented swelling-induced activation of VSOACs (Fig. 4A). Preabsorption of the A1–14 antibody against 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 μg/ml A1–14 antibody, current density measured in hypotonic solution did not differ significantly from that under isotonic conditions. In cells dialyzed with antigen-preabsorbed A1–14 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 (Ex133–146) of ClC-3 were examined in PASMCs (data not shown). After the swelling-induced VSOAC currents reached steady state, application of 1.6 μg/ml Ex133–148 antibody to the bath solution did not cause any noticable 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 Ex133–148 antibody, 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 μg/ml Ex133–148 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 C670–687 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 C670–687 antibody on VSOACs in PASMC 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 μg/ml C670–687 antibody or antigen-preabsorbed C670–687 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 C670–687 antibody for 15 min, hypertonic 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
antigen-preabsorbed C670-687 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 μg/ml C670-687 antibody or antigen-preabsorbed C670-687 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 C670-687 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 μg/ml C670-687 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-CIC-3 antibodys 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 μg/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 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 μg/ml CIC-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.**
cells dialyzed with C670 and hypotonic conditions in control cells as well as in cells intracellularly dialyzed with 10 μg/ml anti-Kv1.1 antibody. Horizontal solid lines above the traces indicate different bath solutions. 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 μg/ml anti-Kv1.1 antibody. Horizontal solid lines indicate the holding potentials greater than and equal to 30 mV at which the currents were measured. B: current 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. *P < 0.05 vs. respective isotonic conditions.

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 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 Cl− > > Cl− and Cl− > Cl−. 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 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 (IKr) is almost completely inactivated at these positive potentials and IKs 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 IKs 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 (IKs). In contrast, when a cell was dialyzed with C670–687 antibody (Fig. 7B), hypotonic cell swelling only increased the slow time-dependent IKs 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 C670–687 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 C670–687 antibody, hypotonic solution caused a similar increase in the slow time-dependent IKs as in control cells; however, activation of the initial time-independent VSOAC currents by hypotonic cell swelling was not observed.
were elicited by 2-s depolarizing pulses to potentials ranging from -1100 to -30 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 μg/ml C670-687 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 C670-687 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 C592–661) 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 PASMCs 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 PASMCs (Fig. 2). A similar molecular mass immunoreactive band was observed using the C670–687 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 C3 antisense oligonucleotides or antisense cRNA significantly reduce the density of a similar molecular mass band using both Alm C592–661 antibody and C670–687 antibody in immunoblots in Hela cells and Xenopus oocytes, respectively (14). Improved specificity of the new antibodies (A1−14, EXT33–145, and C670–687) for ClC-3 is suggested by the relative absence of additional immunoreactive bands in Western blots in cardiac tissue and PASMCs compared with the commercial Alm C592–661 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 C592–661 antibody (8), intracellular dialysis with either C670–687 antibody or A1−14 antibody prevented activation of native VSOACs by hypotonic cell swelling in canine PASMCs 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 CIC-3 exhibit the same blocking effects on native VSOACs is strong evidence that a direct interaction of the antibodies with CIC-3 protein underlies the observed inhibitory effects of the antibodies on native VSOACs. To exclude the possibility that intracellular dialysis with antibodies may nonspecifically 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-CIC-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-CIC-3 antibodies was not caused by nonspecific binding but due to unique reactivity of the anti-CIC-3 antibodies with VSOACs.

Additional evidence for the specificity of the inhibitory effect of the anti-CIC-3 antibodies on VSOACs was obtained from experiments where the effects of C670–687 antibody on the swelling-induced activation of VSOACs and Iks 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 Iks 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-CIC-3 C670–687 antibody did not prevent augmentation of Iks 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 Iks channel nor did it interfere with the signaling pathways mediating swelling-induced augmentation of Iks.

Whereas our data support the role of endogenous CIC-3 as a protein responsible for native VSOACs in guinea pig cardiac myocytes and canine PASMCs, Huang et al. (16) reported that expression of hCIC-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 hCIC-3 in this study might be attributable to possible functional differences in properties of the long isoform of hCIC-3 expressed in these experiments compared with the VSOAC role attributed to expression of the short isoform of CIC-3 (7). It is known that the long isoform of CIC-3 differs significantly from the short CIC-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 analysis with a polyclonal anti-CIC-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 CIC-3 in guinea pig cardiac myocytes and canine PASMCs may serve as a regulator of native VSOACs, the following additional results strongly support CIC-3 as directly responsible for some types of native VSOACs: 1) heterologous expression of gpCIC-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 gpCIC-3 channel (7) and its regulation by protein kinase C and cell volume (5), and 3) CIC-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-CIC-3 antibodies to CIC-3 channel proteins lead to inhibition of VSOACs is unclear. The recently resolved crystal structure of the bacterial CIC channels (9) shows that both the amino and carboxy terminal of CIC are in the vicinity of the channel pore. Thus it seems likely that binding of the anti-CIC-3 antibodies to either the amino terminus or the carboxy terminus of CIC may block the channel pore directly. Alternatively, anti-CIC-3 antibodies may interfere with the gating mechanisms of CIC-3. The ineffectiveness of the extracellular anti-CIC-3 antibody to block native VSOACs is consistent with the CIC 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-CIC-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-CIC-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 CIC-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 CIC-3 Cl⁻ currents in various heterologous expression systems.

The authors thank Linda Ye, Keith Murray, Xiaomin Shen, Yanping Dai, and Phillip Keller for excellent technical assistance and Dr. F. Lamb for providing Clcn3c⁻/⁻ mice.
DISCLOSURES
This study was supported by National Institutes of Health Grants HL-49254 and P20RR-15581.

REFERENCES


