A Serine Residue in ClC-3 Links Phosphorylation–Dephosphorylation to Chloride Channel Regulation by Cell Volume

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abstract In many mammalian cells, ClC-3 volume-regulated chloride channels maintain a variety of normal cellular functions during osmotic perturbation. The molecular mechanisms of channel regulation by cell volume, however, are unknown. Since a number of recent studies point to the involvement of protein phosphorylation/dephosphorylation in the control of volume-regulated ionic transport systems, we studied the relationship between channel phosphorylation and volume regulation of ClC-3 channels using site-directed mutagenesis and patch-clamp techniques. In native cardiac cells and when overexpressed in NIH/3T3 cells, ClC-3 channels were opened by cell swelling or inhibition of endogenous PKC, but closed by PKC activation, phosphatase inhibition, or elevation of intracellular Ca$^{2+}$. Site-specific mutational studies indicate that a serine residue (serine51) within a consensus PKC-phosphorylation site in the intracellular amino terminus of the ClC-3 channel protein represents an important volume sensor of the channel. These results provide direct molecular and pharmacological evidence indicating that channel phosphorylation/dephosphorylation plays a crucial role in the regulation of volume sensitivity of recombinant ClC-3 channels and their native counterpart, $I_{\text{Cl,vol}}$.

key words: ion channels • osmotic stress • signal transduction • protein kinase • protein phosphatase

introduction To avoid excessive alterations of cell volume that may jeopardize structural integrity and a variety of cellular functions, mammalian cells are able to precisely maintain their size in the face of osmotic perturbations through the regulated loss or gain of intracellular ions or other osmolytes (Nilius et al., 1996; Strange et al., 1996; Okada, 1997; Lang et al., 1998). Even under isotonic conditions, volume constancy of any mammalian cell is challenged by the transport of osmotically active substances across the cell membrane and alterations in cellular osmolarity by metabolism (Lang et al., 1998). Thus, the continued operation of cell volume regulatory mechanisms, such as volume-regulated chloride (Cl$^{-}$) currents ($I_{\text{Cl,vol}}$), is required for cell volume homeostasis in many mammalian cells (Nilius et al., 1996; Strange et al., 1996; Okada, 1997). We have recently provided evidence that the channel protein responsible for $I_{\text{Cl,vol}}$ in the heart and many other mammalian cells is encoded by the CIC-3 gene (Duan et al., 1997b; Yamazaki et al., 1998). CIC-3 belongs to the large gene family of CIC Cl$^{-}$ channels that are comprised of 12 putative transmembrane-spanning domains (Kawasaki et al., 1994; Kawasaki et al., 1995; Jentsch, 1996; Schmidt-Rose and Jentsch, 1997). Expressed CIC-3 Cl$^{-}$ channels in oocytes and mammalian cells are strongly inhibited by activation of PKC (Kawasaki et al., 1994, 1995; Duan et al., 1997b) and hypertonic cell shrinkage while they are activated by hypotonic cell swelling (Duan et al., 1997b). Little is currently known, however, about the molecular mechanisms of regulation of $I_{\text{Cl,vol}}$ by cell volume (Okada, 1997; Strange, 1998; Clapham, 1998).

Alternations of cell volume during extra- and intracellular osmotic perturbation trigger a multitude of intracellular signaling events, including various second message cascades, phosphorylation or dephosphorylation of target proteins, as well as altered gene expression (Waldegger et al., 1997a,b; Lang et al., 1998). Cell swelling has been shown to induce protein dephosphorylation, which in turn activates K-Cl cotransport (Jennings and al-Rohil, 1990; Jennings and Schulz, 1991; Bize and Dunham, 1994; Starke and Jennings, 1993) and inhibits Na-K-2Cl cotransport (Klein et al., 1993; Haas et al., 1995; Lytle, 1998). This swelling-induced protein dephosphorylation may be due to decreased kinase activity (Jennings and al-Rohil, 1990; Bize and Dunham, 1994; Gibson and Hall, 1995) and/or increased activities of serine/threonine protein phosphatases (PPs, probably PP1 and PP2A)$^{1}$ (Jennings and

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1Abbreviations used in this paper: BIM, bisindolylmaleimide I-HCl; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; I-V, current–voltage; PDBu, phorbol 12,13-dibutyrate; PP, protein phosphatase.
tions, while inhibition of PKC can activate I

 inhibition of PPs (Hall et al., 1995; Doroshenko, 1998) PKC (Duan et al., 1995; Hardy et al., 1995; Coca-Prados et al., 1998), and cardiac myocytes (Hall et al., 1998). In Ehrlich mouse ascites tumour cells, cell shrinkage causes a rapid increase (174% within 1 min) in PKC activity in the membrane fraction that appears to be involved in the activation of the Na-K-2Cl cotransport after cell shrinkage (Larsen et al., 1994). PKC-dependent phosphorylation is also involved in the cell shrinkage activation of a nonselective conductance in Caco-2 cells (Nelson et al., 1996). In fact, cell swelling and shrinkage have been shown to induce protein dephosphorylation and phosphorylation, respectively, in a variety of cell systems (Grinstein et al., 1992; Palfrey, 1994), including epithelial (Haas et al., 1995) and endothelial (Santell et al., 1993; Klein et al., 1993) cells, erythrocytes (Jennings and al-Rohil, 1990; Jennings and Schulz, 1991; Starke and Jennings, 1993; Lytle, 1998), Ehrlich mouse ascites tumour cells (Larsen et al., 1994; Krakup et al., 1998), and cardiac myocytes (Hall et al., 1995). Therefore, these studies all suggest that phosphorylation/dephosphorylation of proteins (such as ionic channels and transportors) due to altered protein kinase and/or phosphatase activities may be a common process linking changes in cell volume to protein functions.

It is noteworthy that native I_{Cl,vol} in heart and many other tissues is also strongly regulated by phosphorylation/dephosphorylation. Activation of intracellular PKC (Duan et al., 1995; Hardy et al., 1995; Coca-Prados et al., 1996; Bond et al., 1998; Dick et al., 1998) and inhibition of PPs (Hall et al., 1995; Doroshenko, 1998) both strongly inhibit I_{Cl,vol} even under hypotonic conditions, while inhibition of PKC can activate I_{Cl,vol} under isotonic conditions (Coca-Prados et al., 1995, 1996; Dick et al., 1998). To study the volume-sensing mechanism of ClC-3 channels and its potential linkage to channel phosphorylation, we used a variety of approaches from the tight-seal whole-cell voltage-clamp technique as described (Hamill et al., 1981; Duan et al., 1997a,b). To obtain whole-cell current–voltage relations, cells were held at −40 mV and test potentials were applied from −100 to +120 mV for 400 ms in +20-mV increments at an interval of 5 s (voltage-clamp protocol is shown in Fig. 1, top). Current amplitudes were measured at 8 ms after the corresponding voltage step relative to current level and normalized to cell capacitance (pA pF^{−1}). To obtain time-dependent changes in current amplitude before and after different interventions, cells were clamped from a holding potential of −40 mV to hyperpolarizing potential of −100 mV for 100 ms, back to −40 mV for 10 ms, and then to a depolarizing potential of +100 mV for 100 ms (voltage-clamp protocol is shown in Fig. 3, top). The same hyperpolarizing and depolarizing pulses were imposed every 30 s. All results are expressed as mean ± SEM. Statistical comparisons were performed by Student’s t test and a two-tailed probability of <5% was taken to indicate statistical significance.

Solutions and Drugs

Bath and pipette solutions were chosen to facilitate Cl− current recording. The hypotonic (250 mOsm/kg H2O, measured by freezing point depression, Osmomette; Precision Systems Inc.) bath solutions for recording in NIH/3T3 cells contained (mM): 125 NaCl, 2.5 MgCl2, 2.5 CaCl2, 10 HEPES, pH 7.2, [Cl−]o = 135 mM. The isotonic and hypertonic bath solutions were the same as the hypertonic solution except that the osmolality was adjusted to 300 and 350 mOsm/kg H2O, respectively, with mannitol. When experiments were performed with decreased [Cl−]o, iodide (I−) or aspartate (Asp−) was used to replace Cl− at equimolar concentration (110 mM). The pipette (internal) solution for recordings in NIH/3T3 contained (mM): 135 N-methyl-D-glucamine chloride (NMDG-Cl), 2 EGTA, 5 Mg-ATP, 10 HEPES, pH 7.2, [Cl−]i = 135 mM, 300 mOsm/kg H2O using mannitol. The hypotonic (220 mOsm/kg H2O) bath solutions for recording in cardiac myocytes contained (mM): 90 NaCl, 0.8 MgCl2, 1.0 CaCl2, 0.2 CdCl2, 2.0 BaCl2, 0.33 NaH2PO4, 10 tetrathyrammonium-Cl, 10 HEPES, 5.5 glucose, pH 7.4, [Cl−]i = 108 mM. The isotonic bath solutions were the same as the hypertonic solution except that the osmolality was adjusted to 300 mOsm/kg H2O, respectively, with mannitol. When low [Cl−]i was needed, NaI or Na-aspartate was used to replace NaCl at equimolar concentration.

MATERIALS AND METHODS

Site-directed Mutation and Functional Expression of Guinea-Pig Cardiac ClC-3

The serine at position 51 and/or 362 was altered by a S51, S362, and S51 + S362 to an alanine site-specific mutation introduced into gpClC-3 cDNA (Deng and Nickoloff, 1992). The mutation was confirmed by nucleotide sequencing of both strands of the mutated cDNA. NIH/3T3 cells were transiently transfected by electroporation as previously described (Duan et al., 1997b). Each dish was transfected with appropriate combinations of CD8 (a lymphocyte cell surface antigen) in the pH3-CD8 plasmid construct as a marker for transfection (4 μg) and wtClC-3, S31ACIC-3, S362ACIC-3, or S51A + S362A CIC-3 in the pZeoSV vector (20 μg). Transfected cells were identified by their binding to CD8-coated beads (M-450 CD8; Dyna-beads). Cells were subcultured on glass coverslips for electrophysiological recording.

Electrophysiological Recordings

Currents were measured from isolated NIH/3T3 cells or guinea-pig atrial and ventricular myocytes at room temperature (22–24°C) by the tight-seal whole-cell voltage-clamp technique as described (Hamill et al., 1981; Duan et al., 1997a,b). To obtain whole-cell current–voltage relations, cells were held at −40 mV and test potentials were applied from −100 to +120 mV for 400 ms in +20-mV increments at an interval of 5 s (voltage-clamp protocol is shown in Fig. 1, top). Current amplitudes were measured at 8 ms after the corresponding voltage step relative to current level and normalized to cell capacitance (pA pF^{−1}). To obtain time-dependent changes in current amplitude before and after different interventions, cells were clamped from a holding potential of −40 mV to hyperpolarizing potential of −100 mV for 100 ms, back to −40 mV for 10 ms, and then to a depolarizing potential of +100 mV for 100 ms (voltage-clamp protocol is shown in Fig. 3, top). The same hyperpolarizing and depolarizing pulses were imposed every 30 s. All results are expressed as mean ± SEM. Statistical comparisons were performed by Student’s t test and a two-tailed probability of <5% was taken to indicate statistical significance.
(90 mM). The pipette (internal) solution for recordings in cardiac myocytes contained (mM): 108 NMDG-Cl, 2 EGTA, 5 Mg-ATP, 10 HEPES, pH 7.4. [Cl]i = 108 mM, 290 mOsm/kg H2O using mannitol. In some experiments, cell diameters were measured continuously using a video edge detector (Crescent Electronics) and cell volumes were calculated assuming a simple spherical geometry. All chemicals were from Sigma Chemical Co. Phorbol 12,13-dibutyrate (PDBu), bisindolylmaleimide I HCl (BIM), okadaic acid, and calyculin A were obtained from Calbiochem Corp. and prepared as stock solutions of 1 or 10 mM in dimethyl sulfoxide and added to known volume of superfusion solutions to produce the desired concentrations.

RESULTS

Under isotonic (300 mOsm/kg H2O) symmetrical Cl− (135 mM) conditions, wtClC-3–transfected cells generated basally active outwardly rectifying whole-cell currents (Fig. 1 A, a) with a mean current density of $442 \pm 38 \text{pA} \cdot \text{pF}^{-1}$ at $+80 \text{mV}$ and $-253 \pm 27 \text{pA} \cdot \text{pF}^{-1}$ at $-80 \text{mV}$ and a mean reversal potential of $-1.8 \pm 0.3 \text{mV}$ ($n = 6$). Exposure of these cells to hypotonic solutions (250 mOsm/kg H2O, 17% hypotonic) for $>2 \text{min}$ caused significant cell swelling and increased the membrane current densities to $946 \pm 56 \text{pA} \cdot \text{pF}^{-1}$ at $+80 \text{mV}$ and $-595 \pm 46 \text{pA} \cdot \text{pF}^{-1}$ at $-80 \text{mV}$ ($n = 6$) due to an increase in the number of active channels (Strange et al., 1996; Duan et al., 1997a,b) (Fig. 1 A, b). These results indicate that under basal isotonic conditions, most expressed ClC-3 channels remain in a closed state that can be activated by hypotonic cell swelling, suggesting the possible existence of an endogenous cytosolic inhibitor under isotonic conditions (Krick et al., 1991; Kawasaki et al., 1995). Activation of PKC by PDBu (100 nM) under hypotonic conditions strongly inhibited the currents (Fig. 1 A, c) in a voltage-independent fashion as previously described (Duan et al., 1997b), while hypotonic solutions induced a similar increase in cell volume in control (129 ± 5.4%, $n = 8$) and PDBu-containing solutions (122 ± 2.0%, $n = 7$, $P = \text{NS}$). Downregulation of endogenous PKC by exposure of wtClC-3–transfected NIH/3T3 cells to PDBu (1 µM) for $>24 \text{h}$ (Duan et al., 1995; Pears and Goode, 1997) not only abolished the inhibition of wtClC-3 currents by acute application of PDBu (Fig. 1 B, c), but also, surprisingly, changed the volume sensitivity of these channels. In downregulated cells under isotonic conditions, most channels were constitutively open with a mean current density of $1,009 \pm 92 \text{pA} \cdot \text{pF}^{-1}$ at $+80 \text{mV}$ and $-678 \pm 60 \text{pA} \cdot \text{pF}^{-1}$ at $-80 \text{mV}$ ($n = 4$) (Fig. 1 B, a) and subsequent hypotonic cell swelling failed to significantly increase current densities (Fig. 1 B, b) in these cells (1,039 ± 91 pA pF−1 at +80 mV and −701 ± 70 pA pF−1 at −80 mV, $P = \text{NS}$). Inhibition of endogenous PKC in wtClC-3–transfected cells by acute application of BIM (100 nM), a highly selective PKC inhibitor (Toulecc et al., 1991), also dramatically increased membrane Cl− current densities under isotonic conditions (Fig. 1 C, a and b) and abolished further activation of expressed channels by cell swelling (Fig. 1 C, c). The endogenous Cl− currents in NIH/3T3 cells, while also volume sensitive, contribute very little to the results described above (Duan et al., 1997b; also see Fig. 6, A and B). These results strongly suggest that endogenous PKC in these NIH/3T3 cells is a strong cytosolic inhibitor of ClC-3 channels and that relief of PKC inhibition may be linked to hypotonic-induced opening of the channel. To further test this hypothesis, we performed similar experiments in isolated guinea-pig atrial and ventricular cells from which the ClC-3 gene was originally cloned (Duan et al., 1997b). As shown in Fig. 2, both the basally active and swelling-activated currents in atrial (Fig. 2 A) and ventricular (Fig. 2 B) myocytes were also strongly inhibited by PKC activation. Identical to the cloned gpClC-3 channel expressed in NIH/3T3 cells (Duan et al., 1997b), these cell swelling- and PKC-sensitive currents in both atrial (Fig. 2 C) and ventricular (Fig. 2 D) myocytes had an anion selectivity of $1^- > Cl^- >> Asp^-$. Consistent with our observations in NIH/3T3 cells, BIM-induced inhibition of endogenous PKC also activated native $I_{\text{Cl,cell}}$ in both atrial (Fig. 3 A, b) and ventricular (Fig. 3 B, b) myocytes under isotonic conditions and prevented further activation by subsequent hypotonic cell swelling (Fig. 3, A, c and B, c). As shown in Fig. 3 C, the swelling- and BIM-induced currents were inhibited by extracellular 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) (100 µM, Fig. 3 C, a and b) and ATP (10 mM, Fig. 3 C, c) in a characteristic voltage-dependent manner that closely resembles the inhibition of native $I_{\text{Cl,cell}}$ by these compounds in a wide variety of cells (Duan et al., 1995; Vandenberg et al., 1994; Nilius et al., 1996; Strange et al., 1996; Okada, 1997; Yamazaki et al., 1998) and ClC-3 currents in oocytes and mammalian cells (Kawasaki et al., 1994; Duan et al., 1997b). These results support the idea that PKC phosphorylation and dephosphorylation of both the wtClC-3 and native channel protein play a crucial role in channel regulation by changes in cell volume.

In intact cells, processes that are reversibly controlled by protein phosphorylation require not only a protein kinase but also a protein phosphatase (Hunter, 1995). The net level of protein phosphorylation depends on the balance of kinase and phosphatase activities (Cohen, 1992), and both protein kinases and phosphatases have been reported to be subject to regulation by cell volume (Jennings and al-Rohil, 1990; Jennings and Schulz, 1991; Starke and Jennings, 1993; Waldegger et al., 1997a; Lang, 1998). In fact, inhibition of serine/threonine protein phosphatases by calyculin A in NIH/3T3 cells causes not only a marked increase in protein phosphorylation in both cytosolic and insoluble cellular fractions, but also a reversible cell shape change. 

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(Chartier et al., 1991). To further test the hypothesis that a balance between channel protein phosphorylation and dephosphorylation may be the key regulatory event responsible for ClC-3 channel regulation by cell volume in the face of osmotic perturbation, we studied the effects of two highly potent serine/threonine protein phosphatase inhibitors, okadaic acid (Cohen et al., 1990) and calyculin A (Ishihara et al., 1989), on wtClC-3 channels expressed in NIH/3T3 cells. As shown in Fig. 4, both okadaic acid (100 nM, A) and calyculin A (20 nM, B) not only inhibited basally active wtClC-3 channels under isotonic conditions, but also prevented hypotonic cell-swelling activation of these channels. Similar results were observed in four (okadaic acid) and five (calyculin A) different cells in which wtClC-3 were stably or transiently transfected. Both basally and swelling-activated ClC-3 channels were always strongly inhibited by either okadaic acid or calyculin A, when added before or after induction of cell swelling, indicating that PPs are continuously involved in channel regulation and the balance of PKC-PP activity is constantly regulated by cell volume.
The apparent link between PKC phosphorylation–dephosphorylation and cell swelling–induced activation of ClC-3 channels prompted a consideration of putative protein PKC phosphorylation sites as potential volume sensors (Kawasaki et al., 1994; Duan et al., 1997b; GenBank accession #U83464). One is serine 51 near the amino terminus (Arg-Arg-Ile-Asn-Ser51-Lys-Lys) and the other is serine 362 within the cytoplasmic loop between transmembrane domains D7 and D8 (Arg-Arg-Lys-Ser362-Thr-Lys). We tested the hypothesis that the inhibition, by PKC activators and phosphatase inhibitors, and the activation, by hypotonic cell swelling and PKC inhibitors, of ClC-3 channels may be due to direct phosphorylation–dephosphorylation of S51 or S362. Serine 51 and/or serine 362 were changed to alanines and three mutants were generated: single mutants S51A, S362A, and double mutant S51A + S362A (Fig. 5 A). Results of whole-cell patch-clamp recording from these three ClC-3 mutants transfected into 3T3 cells are shown in Fig. 5, B–D. Cells were exposed either to isotonic (a), hypotonic (b), or hypotonic PDBu 100 nM (c) conditions. (C and D) I–V relationship of whole-cell currents in atrial (C) and ventricular (D) myocytes before and after [Cl−], was replaced by equimolar (90 mM) I− or Asp− (ECl− = +46 mV). I− and Asp− substitution of [Cl−], shifted the reversal potential of ICl.vol in atrial myocytes (C) from −20 ± 0.5 to −15.3 ± 1.3 and +39.5 ± 1.7 mV (n = 6), respectively, and of ICl.vol in ventricular myocytes (D) from −32 ± 0.9 to −13.8 ± 2.4 and +41.2 ± 5.9 mV (n = 5), respectively. Voltage-clamp protocol is the same as shown in Fig. 1, top.
current density of $952 \pm 41 \text{ pA pF}^{-1}$ at $+80 \text{ mV}$ and $-606 \pm 71 \text{ pA pF}^{-1}$ at $-80 \text{ mV}$, $P = \text{NS}$). Similar results were obtained from S51AGIC-3–transfected cells. Most channels were open even under isotonic conditions and generated a large constitutively active $I_{\text{ClC}-3}$ (Fig. 5 C, a, mean current density of $930 \pm 18 \text{ pA pF}^{-1}$ at $+80 \text{ mV}$ and $-608 \pm 22 \text{ pA pF}^{-1}$ at $-80 \text{ mV}$, $n = 7$) that was no longer responsive to cell swelling (Fig. 5 C, b, mean
current density of 971 ± 27 pA pF⁻¹ at +80 mV and
−650 ± 23 pA pF⁻¹ at −80 mV, P = NS, compared with
isotonic conditions) or PDBu (Fig. 5 C, d, mean cur-
tent density of 907 ± 31 pA pF⁻¹ at +80 mV and
−348 ± 31 pA pF⁻¹ at −80 mV, P < 0.05 compared with
isotonic and hypotonic conditions). As shown in Fig. 5 D, how-
ever, the S362AClC-3 mutant yielded a channel with an
intermediate responsiveness to cell swelling and PKC.

The mean current densities of S362AClC-3 under iso-
tonic conditions were 610 ± 42 pA pF⁻¹ at +80 mV and
−348 ± 31 pA pF⁻¹ at −80 mV (n = 6), which are signifi-
cantly higher (P < 0.05) than the mean current densi-
ties of wtClC-3 under the same isotonic conditions. Ex-
posure of these S362AClC-3–transfected cells to hypo-
tonic solutions caused a further increase in current
densities (912 ± 90 pA pF⁻¹ at +80 mV and −468 ± 37
pA pF⁻¹ at −80 mV, n = 6, P < 0.05 compared with iso-
tonic conditions). Activation of PKC by PDBu under
hypotonic conditions caused significantly less inhibi-
tion of S362AClC-3 current than that of wtClC-3 (55 ±
7% inhibition of S362AClC-3 currents [n = 6] vs. 81 ±
3% inhibition of wtClC-3 currents [n = 6] at +80 mV,
P < 0.01). We also performed blinded experiments in
which coded NIH/3T3 cells transiently transfected with
CD8 alone (control), wtClC-3, S51AClC-3, S362AClC-3,
and S51A:S362AClC-3 mutants were used in a ran-
domized fashion to study the response of expressed
currents to hypotonic cell swelling and PKC activation.

Fig. 6 compares the mean current densities of cells
transiently transfected with CD8 alone (control), wt-
ClC-3, S51AClC-3, S362AClC-3, and S51A+S362AClC-3
mutants at +80 mV under isotonic (A), hypotonic (B),
and hypotonic PDBu 100 nM (C) conditions, respec-
tively. While only very small currents could be detected
from CD8-transfected cells under isotonic and hypo-
tonic conditions with similar densities as reported be-
fore (Duan et al., 1997b), it is very clear that wtClC-3
and all three ClC-3–mutant transfected cells elicited
significantly larger basally active outwardly rectifying
currents under isotonic conditions. Wild-type ClC-3
channels were activated by cell swelling and inhibited
by PKC activation, as previously shown (Fig. 1 A, a).
Both S51AClC-3 and S51A+S362AClC-3 channels were
constitutively opened under isotonic conditions and
thus generated significantly larger “basal” currents than
wtClC-3 (Fig. 6 A). These phenotypes exhibited no sig-
nificant response to changes in cell volume (Fig. 6 B)
or activation of PKC (Fig. 6 C). Basal S362AClC-3 cur-
tents were larger than wtClC-3 (P < 0.05) but smaller
than S51AClC-3 and S51A+S362AClC-3 (P < 0.05)
(Fig. 6 A). This phenotype had intermediate response
to cell volume (Fig. 6 B) and PKC (Fig. 6 C). As shown
in Fig. 7 A, a, hypertonic (350 mOsm/kg H₂O) cell
shrinkage also failed to inhibit S51AClC-3 channels
(898 ± 32 pA pF⁻¹ under isotonic conditions vs. 869 ±
48 pA pF⁻¹ under hypertonic conditions, n = 5, P = NS). However, extracellular nucleotides (ATP, 10 mM, Fig. 7 A, b), DIDS (100 μM, Fig. 7 A, c), and tamoxifen (10 μM, Fig. 7 A, d) all blocked S51ACIC-3 with a characteristic voltage dependence closely resembling their effects on wtCIC-3 (Duan et al., 1997b; Yamazaki et al., 1998). The anion selectivity of these CIC-3 mutants were examined (Fig. 7 B). Table I summarizes the shifts in the reversal potentials of native ICl.vol in guinea-pig atrial and ventricular myocytes and wild-type and mutant CIC-3 channels in NIH/3T3 cells induced by I⁻ or Asp⁻ and their relative permeabilities. The permeability ratios were calculated from the shifts using the modified Goldman-Hodgkin-Katz equation (Hille, 1992) for monovalent anion substitutions. All three mutants of CIC-3 had a similar permeability ratio to I⁻ or Asp⁻ with respect to Cl⁻ and an identical anion-selectivity of I⁻ > Cl⁻ > Asp⁻ as native ICl.vol and wtCIC-3.

It has been reported that rat CIC-3 channels may be inhibited by increases in intracellular Ca²⁺ when expressed in Chinese hamster ovary cells (Kawasaki et al., 1995). Similarly, exposure of guinea-pig cardiac wtCIC-3–transfected cells to the Ca²⁺ ionophore, ionomycin (1 μM), also caused a dramatic inhibition of wtCIC-3 currents under hypotonic conditions (Fig. 8 A, c and d). As shown in Fig. 6 B, however, the inhibition of gpCIC-3

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**Figure 5.** Effects of PKC on mutant S51A, S362A, and S51A + S362A gpCIC-3 transiently expressed in NIH/3T3 cells. (A) Transmembrane topology model for CIC Cl⁻ channels (Schmidt-Rose and Jentsch, 1997) and the location of mutated residues. (B–D) Whole-cell currents recorded from three mutants of CIC-3 under isotonic (a), hypotonic (b), and hypotonic PDBu 100 nM (c) conditions, respectively; d show corresponding mean I–V curves from different cells transfected with S51A+S362ACIC-3 (B, d, n = 5), S51ACIC-3 (C, d, n = 7), and S362ACIC-3 (D, d, n = 6), respectively.
currents by ionomycin was prevented by inhibition of endogenous PKC when cells were pretreated with BIM (100 nM). These results suggest that inhibition of gpClC-3 channels by an increase in intracellular Ca\(^{2+}\) may be due to a Ca\(^{2+}\)-sensitive PKC-phosphorylation–mediated mechanism. This is different from rat ClC-3 channels (Kawasaki et al., 1995) in which the Ca\(^{2+}\) inhibition of ClC-3 channels in inside-out membrane patches from Chinese hamster ovary cells was reported to be phosphorylation independent (Kawasaki et al., 1995). The reason for this discrepancy is unclear. Both Ca\(^{2+}\)-sensitive (PKCa) and -insensitive (PKCb and PKCe) PKC isozymes are abundantly expressed in NIH/3T3 cells (Szallas et al., 1994), and BIM inhibits all PKCa, β\(_{1b}\), β\(_{II}\), γ, δ, and ε isozymes (Gekeler et al., 1996; Toullec et al., 1991). Thus, it is possible that both Ca\(^{2+}\)-sensitive and -insensitive PKC isozymes can phosphorylate the ClC-3 protein and cause similar conformational changes to close the channel. To further examine whether the inhibition of gpClC-3 channels by intracellular Ca\(^{2+}\) is dependent on PKC phosphorylation of the channel, we performed further experiments to study the response of expressed mutant S51AClC-3 channel to ionomycin. While the properties of S51AClC-3 channels under isotonic and hypotonic conditions (Fig. 8 C, a and b) were identical to those shown in Fig. 5 C, a and b, respectively, ionomycin failed to inhibit S51AClC-3 channels (Fig. 8 C, c and d), confirming the involvement of amino-terminal PKC-phosphorylation in the Ca\(^{2+}\) inhibition of ClC-3 channels.

**Discussion**

In this study, we examined the molecular mechanism responsible for the activation of volume-regulated Cl\(^{-}\) channels by hypotonic cell swelling using both cloned guinea-pig cardiac ClC-3 expressed in NIH/3T3 cells and native I\(_{\text{Cl,vol}}\) currents in guinea-pig atrial and ventricular myocytes. Our functional and mutational studies of the ClC-3 gene product indicate that the activation of ClC-3 channels during hypotonic-induced cell swelling is attributable to relief of endogenous PKC inhibition of these channels caused by cell swelling–induced dephosphorylation of a serine residue within the amino terminus of the channel protein. Thus, these results provide an important new clue into the molecular link between changes in cell volume, protein phosphorylation–dephosphorylation, and channel function.

Protein phosphorylation or dephosphorylation is a common rapid and reversible means of transducing signals from the extracellular environment to many cellular responses (Witters, 1990; Hunter, 1995). It should not be surprising that cells are able to use these rapid and reversible means to control their sizes. How changes in cell volume are linked to changes in activity of protein kinases or phosphatases is still not clear. One of the most acute signaling events triggered by osmotic challenge may be dilution or concentration of cellular constituents including proteins leading to changes in intracellular macromolecular crowding and confinement, which may profoundly alter kinase-phosphatase activities (Fulton, 1982; Jennings and al-Rohil, 1990; Jennings and Schulz, 1991; Minton et al., 1992; Starke and Jennings, 1993; Garner and Burg, 1994). Alternatively, slower subacute signaling events caused by cell swelling or shrinkage may be related to changes in the synthesis of second messengers. For example, exposure of mammalian and plant cells to acute hyperosmotic stress stimulates rapid synthesis of phosphatidylinositol-3,5-bisphosphate, a new phosphoinositide second messenger in the phospholipase C–PKC cascade that may associate with the cytoskeleton (Dove et al., 1994, 1997). Finally, the slowest and long-term intracellular signaling events involved in continuous volume regulation may be due to alterations in gene expression of second messengers (Waldegger et al., 1997a; Waldegger and Lang, 1998). The exact interaction between cell volume and elements of intracellular signaling and detailed intermediate processes of how PKC and PP activities may be regulated by cell volume requires further study.

Our data suggests that ClC-3 channels may exist in either a closed phosphorylated state or an active dephos-
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PKC phosphorylation of the NH₂ terminus of CIC-3 channel may cause a crucial change in channel conformation and close the channel pore. Combined with data from our previous studies and those of other laboratories (Li et al., 1989; Jennings and al-Rohil, 1990; Witters, 1990; Garner and Burg, 1994; Duan et al., 1995, 1997a,b; Coca-Prados et al., 1995, 1996; Strange et al., 1996; Dove et al., 1997; Waldegger et al., 1997a), we propose that CIC-3 channels are continuously controlled by a volume-sensitive phosphorylation–dephosphorylation reaction mediated by PKC (both Ca²⁺-sensitive and -insensitive isozymes) and PPs (probably PP1 and PP2A). Under isotonic conditions, a balance of basal PKC and PP activities usually keeps most CIC-3 channels in a phosphorylated closed state and only few channels are in the dephosphorylated open state. These few active channels generate a “basal” current (Duan et al., 1992, 1995, 1997a,b; Liu et al., 1993; Coca-Prados et al., 1995; Voets et al., 1996; Dick et al., 1998). Under hypotonic conditions, PKC activity is diminished due possibly to dilution (Jennings and al-Rohil, 1990; Garner and Burg, 1994), redistribution or alteration in PKC or PP activity (Jennings and Schulz, 1993; Starke and Jennings, 1993; Palfrey, 1994; Lytle, 1998); CIC-3 channels become dephosphorylated and more channels open, producing a larger macroscopic current (Duan et al., 1995, 1997a,b; Strange et al., 1996). Under hypertonic conditions, PKC activity may be increased (Jennings and al-Rohil, 1990; Larsen et al., 1994; Garner and

Figure 7. Pharmacology and ion selectivity of CIC-3 mutants in NIH/3T3 cells. (A) Representative whole-cell recording of S51A CIC-3 currents under isotonic conditions and in the presence of extracellular hypertonicity (a), ATP (b), DIDS (c), and tamoxifen (d). While hypertonic cell shrinkage failed to inhibit S51A CIC-3 current, extracellular ATP (10 mM), DIDS (100 μM), and tamoxifen (TMX, 10 μM) all inhibited S51A CIC-3 currents in a characteristic voltage-dependent manner identical to their inhibitory effects on the wtCIC-3 channels (Duan et al., 1997b). (B) I–V relationship of whole-cell currents in NIH/3T3 cells transfected with S51A + S362A CIC-3 (a), S51A CIC-3 (b), S362A CIC-3 (c under isotonic conditions and d under hypotonic conditions) before and after (Cl⁻), was replaced by equimolar (110 mM) I⁻ or Asp⁻ (EC₅₀ = +42.8 mV). I⁻ and Asp⁻ substitution of [Cl⁻], shifted the reversal potential of S51A + S362A CIC-3 current (a) from −1.0 ± 1.2 to −14.2 ± 1.1 and +43.8 ± 3.2 mV (n = 4), respectively, of S51A CIC-3 current (b) from −3.8 ± 0.3 to −14.9 ± 2.6 and +37.7 ± 1.6 mV (n = 3), respectively, of S362A CIC-3 current under isotonic condition (c) from −2.3 ± 0.8 to −17.0 ± 2.3 and +41.0 ± 2.6 mV (n = 4), respectively, and S362A CIC-3 current under hypotonic condition (d) from −2.2 ± 1.1 to −14.5 ± 2.1 and +38.1 ± 1.5 mV (n = 3), respectively. Voltage-clamp protocol is the same as shown on the top of Fig. 1.
and PP activity may be diminished (Palfrey, 1994; Lytle, 1998), thus more channels become phosphorylated and close. Therefore, it is proposed that serine 51, a putative PKC phosphorylation site near the NH₂ terminus of CIC-3, may represent an important volume sensor of the channel that directly links channel activity to alterations in intracellular PKC-PP activity.

PKC activation has been previously reported to inhibit voltage-dependent Cl\(^-\) conductances in hippocampal pyramidal cells (Madison et al., 1986), resting Cl\(^-\) conductance in vascular smooth muscle cells (Sagusa and Kokubun, 1988), skeletal muscle cells (Brinkmeier and Jockusch, 1987), CIC-1 in skeletal muscle and HEK cell expression system (Rosenbohm et al., 1995), and the hyperpolarization-activated CIC-2 Cl\(^-\) channel that is also regulated by cell volume (Staley, 1994; Staley et al., 1996; Frisch and Edelman, 1996, 1997). Consistent with our finding in native guinea-pig atrial and ventricular myocytes and cloned CIC-3–transfected NIH/3T3 cells, PKC activation inhibits I\(_{\text{Cl,vol}}\) and outwardly rectifying chloride channel in rabbit atrial myocytes (Duan et al., 1995), MDR1-transfected 3T3

### Table I

|                | \(\Gamma^*\), Vr shift | \(P_x/P_{\text{Cl}}\) | Asp\(^-\), Vr shift | \(P_{\text{Asp}}/P_{\text{Cl}}\) | \(n\) |
|----------------|------------------------|-----------------------|---------------------|-----------------------------|------|
| G-P atrial cells | -11.3 ± 0.6 \(mV\) | 1.27 ± 0.03           | 43.6 ± 1.5 \(mV\)  | 0.15 ± 0.01 \(P_{\text{Cl}}\) | 6    |
| G-P ventricular cells | -10.7 ± 1.0 \(mV\) | 1.24 ± 0.04           | 44.4 ± 2.1 \(mV\)  | 0.15 ± 0.01 \(P_{\text{Cl}}\) | 5    |
| wtCIC-3        | -9.1 ± 1.2 \(mV\)     | 1.16 ± 0.06           | 46.7 ± 2.2 \(mV\)  | 0.16 ± 0.03 \(P_{\text{Cl}}\) | 4    |
| S52A+S62A CIC-3| -13.2 ± 1.0 \(mV\)    | 1.36 ± 0.10           | 44.8 ± 3.0 \(mV\)  | 0.15 ± 0.02 \(P_{\text{Cl}}\) | 4    |
| S51ACIC-3      | -11.1 ± 3.6 \(mV\)    | 1.28 ± 0.18           | 41.6 ± 2.3 \(mV\)  | 0.16 ± 0.01 \(P_{\text{Cl}}\) | 3    |
| S62A CIC-3     | -12.3 ± 2.9 \(mV\)    | 1.33 ± 0.16           | 40.3 ± 1.9 \(mV\)  | 0.17 ± 0.01 \(P_{\text{Cl}}\) | 3    |

Values are means ± SEM; Vr, reversal potential; \(P_x/P_{\text{Cl}}\), permeability ratio of ion X with respect to Cl\(^-\); \(n\), number of cells.

Burg, 1994; Nelson et al., 1998; Dove et al., 1997; Waldegger et al., 1997a,b) and PP activity may be diminished (Palfrey, 1994; Lytle, 1998), thus more channels become phosphorylated and close. Therefore, it is proposed that serine 51, a putative PKC phosphorylation site near the NH₂ terminus of CIC-3, may represent an important volume sensor of the channel that directly links channel activity to alterations in intracellular PKC-PP activity.

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**Figure 8.** Effects of increase in intracellular Ca\(^{2+}\) on wild-type and mutant S51ACIC-3 currents in NIH/3T3 cells. (A) Ionomycin (1 \(\mu\)M) inhibited wtCIC-3 currents under hypotonic conditions in the presence of 2.5 mM external Ca\(^{2+}\). Representative whole-cell currents recorded under isotonic, hypotonic, and hypotonic ionomycin 1 \(\mu\)M conditions and the mean I–V curves of each (\(n = 4\)) are shown in a–d, respectively. (B) Pretreatment of wtCIC-3 transfected NIH/3T3 cell with BIM (100 nM) under isotonic condition (a) abolished the upregulation effect by hypotonic cell swelling (b) and inhibitory effect by ionomycin (c). d shows mean I–V curves from six different cells. (C) Mutation at the amino-terminal PKC-phosphorylation site of CIC-3 (S51ACIC-3) also abolished the upregulation effect by hypotonic cell swelling (b) and inhibitory effect by ionomycin (c). Similar results were obtained from five different cells and the average I–V curves under each condition are shown in d.
cells (Hardy et al., 1995), human airway epithelial cells (Li et al., 1989), and canine visceral smooth muscle cells (Dick et al., 1998). Inhibition of PPs has also been shown to inhibit I_C,vol in chick heart cells (Hall et al., 1995) and bovine chromaffin cells (Doroshenko, 1998). Recent studies in human nonpigmented ciliary epithelial cells and canine colonic smooth muscle cells have also shown that PKC inhibitors isosmotically up-regulated I_C,vol (Coca-Prados et al., 1995, 1996; Dick et al., 1998). Dephosphorylation and cell swelling also activate a voltage-gated Cl− channel in ascidian embryos (Villaz et al., 1995). Inhibition of PKC also activates the volume-sensitive ClC-2 channel in human intestinal T84 epithelial cells (Fritsch and Edelman, 1996). Our results provide direct molecular and pharmacological evidence indicating that channel phosphorylation–dephosphorylation plays a crucial role in regulation of volume sensitivity of ClC-3 channels and native I_C,vol. Therefore, these data provide further evidence that protein kinases and phosphatases may be secondary mediators of a subset of cellular responses to cell volume changes that directly control the function of proteins such as ClC-3 channels.

It has also been reported, however, that I_C,vol in some tissues is either stimulated by phorbol esters, presumably through activation of PKC, or not regulated by PKC activators and inhibitors (Jackson and Strange, 1993; Szücs et al., 1996; Miwa et al., 1997; reviewed by Strange et al., 1996; Okada, 1997; and Strange, 1998). While most of these studies did not directly measure PKC activity, Miwa et al. (1997) reported that phorbol ester TPA (12- O-tetradecanoylphorbol-13-acetate) still had no effect on I_C,vol in human epidermoid KB cells even though activation of PKC by this compound could be biochemically proven. The reason for these discrepancies is not clear. It is possible, however, that the intrinsic response of cells to phorbol esters in terms of activation of different PKC isoforms may vary from cell to cell (Nishizuka, 1988; Hug and Sarre, 1993) and the phosphorylation of ClC-3 chloride channel may be mediated by specific PKC isoforms. Another possibility is that the so-called “PKC activators” (phorbol esters) and “PKC inhibitors” may also act on unknown protein kinases other than PKC. It may be possible that the kinase involved in regulation of ClC-3 is not simply PKC but another serine/threonine kinase acting at the same S51. Waldegger et al. (1997a) have recently cloned a volume-regulated serine/threonine protein kinase designated h-sgk, which is upregulated by hypertonic cell shrinkage and depressed by hypotonic cell swelling. h-sgk has 50% homology throughout its catalytic domain with PKC and is widely expressed in many tissues. However, gene transcription and translation of h-sgk may be too slow to account for the change in I_C,vol. Simple biochemical experiments or attempts to measure activities or translocations of PKC or PKC isoforms may not be sufficient to provide a definitive answer to the complex question whether or not PKC is in fact activated by cell shrinkage and inhibited by swelling. Obviously, more extensive experiments will be needed to detect rapid but more subtle changes in PKC or other protein kinases or phosphatases near the membrane during volume alterations. Although our mutation experiments on the consensus PKC phosphorylation sites provide strong evidence supporting the role of PKC in the volume regulation of the channel, direct measurement of changes in kinase and/or phosphatase activity and biochemical evidence for phosphorylation of ClC-3 during alterations of cell volume are needed. It should also be pointed out that consensus phosphorylation sites of a protein can be promiscuous and mutations can alter the conformation of the protein independent of phosphorylation. In some cells, the mechanism of channel activation by cell swelling may be more complicated than described here since it is also possible that the volume-regulated Cl− channels in some native cells may actually be composed of heterodimers; e.g., ClC-3 along with other ClC subunits (Lorenz et al., 1996). Whether a non-PKC-regulated but volume-sensitive ClC-3 isoform, or heteromultimer, or other members of the ClC-3/CIC-4/CIC-5 subbranch (Jentsch, 1996) are also involved in cell-volume regulation and account for I_C,vol in other cell types needs further investigation.

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