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$_{Cl.vol}$.

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$_{Cl.vol}$), 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$_{Cl.vol}$ in the heart and many other mammalian cells is encoded by the ClC-3 gene (Duan et al., 1997b; Yamazaki et al., 1998). ClC-3 belongs to the large gene family of 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 ClC-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$_{Cl.vol}$ 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

$^{1}$Abbreviations 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.
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) and cardiac myocytes (Hall et al., 1995). Therefore, these studies all suggest that phosphorylation/dephosphorylation of proteins (such as ionic channels and transporters) 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 $I_{\text{Cl,vol}}$ 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 $I_{\text{Cl,vol}}$ even under hypotonic conditions, while inhibition of PKC can activate $I_{\text{Cl,vol}}$ under isotonic conditions (Coca-Prados et al., 1995, 1996; Dick et al., 1998). To study the volume-sensing mechanism of CIC-3 channels and its potential linkage to channel phosphorylation, we used a variety of approaches from the tight-seal whole-cell voltage-clamp study of physiological agents on native and cloned channels to analysis of the functional expression of wild-type and site-directed mutants of CIC-3 channels in NIH/3T3 cells. All of the results in this study are consistent with the identification of the volume sensor as a distinct serine residue in a consensus PKC-phosphorylation site in the intracellular amino terminus of CIC-3 chloride channel. These results thus provide a new structural link between protein function (channel activity) and alterations in phosphorylation–dephosphorylation in response to changes in cell volume during osmotic perturbations.

**MATERIALS AND METHODS**

**Site-directed Mutation and Functional Expression of Guinea-Pig Cardiac CIC-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 gpCIC-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-C8 plasmid construct as a marker for transfection (4 μg) and wtCIC-3, S1AICC-3, S362AICC-3, 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 zero 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 hypotonic solution except that the osmolarity 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, 350 mOsm/kg H₂O respectively, with 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 tetraethylammonium-Cl⁻, 10 HEPES, 5.5 glucose, pH 7.4, [Cl⁻]o = 108 mM. The isotonic bath solutions were the same as the hypotonic solution except that the osmolarity was adjusted to 300 mOsm/kg H₂O, respectively, with mannitol. When low [Cl⁻]o was needed, NaI or Na-aspartate was used to replace NaCl at equimolar concentration.
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 ± 38 pA pF−1 at +80 mV and −253 ± 27 pA pF−1 at −80 mV and a mean reversal potential of −1.8 ± 0.3 mV (n = 6). Exposure of these cells to hypotonic solutions (250 mOsm/kg H2O, 17% hypotonic) for >2 min caused significant cell swelling and increased the membrane current densities to 946 ± 56 pA pF−1 at +80 mV and −595 ± 46 pA pF−1 at −80 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 = NS). Downregulation of endogenous PKC by exposure of wtClC-3–transfected NIH/3T3 cells to PDBu (1 μM) for >24 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 ± 92 pA pF−1 at +80 mV and −678 ± 60 pA pF−1 at −80 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 = NS). Inhibition of endogenous PKC in wtClC-3–transfected cells by acute application of BIM (100 nM), a highly selective PKC inhibitor (Touleec 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–induced and PKC-sensitive currents in both atrial (Fig. 2 C) and ventricular (Fig. 2 D) myocytes had an anion selectivity of $I^{-} < Cl^{-} >> Asp^{-}$. Consistent with our observations in NIH/3T3 cells, BIM-induced inhibition of endogenous PKC also activated native I Cl vol 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 Cl vol 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.
Volume Sensor in the ClC-3 Chloride Channel (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.

**Figure 1.** Relations between PKC activity and volume regulation of wtClC-3 in NIH/3T3 cells. (A) PKC activation inhibited both basally active and hypotonic cell-swelling-induced I_{ClC3}. When overexpressed in NIH/3T3 cells, only a small portion of the ClC-3 channels were active under isotonic conditions (a). Subsequent exposure of the same cell to hypotonic solutions caused a further increase in current amplitude (b). Activation of PKC by PDBu (100 nM) under hypotonic conditions caused a closure of most channels (c). Mean current-voltage (I–V) curves from six cells under isotonic (○), hypotonic (●), and hypotonic PDBu 100 nM (■) conditions are shown in d. (B) Downregulation of endogenous PKC by exposure of the wtClC3-transfected NIH/3T3 cells to PDBu 1 μM for >24 h not only abolished the inhibition of I_{ClC3} by acute application of PDBu (100 nM), but also changed the sensitivity to changes in osmolality. (a) Representative current traces recorded from the PDBu-pre-treated ClC-3 stably transfected cells under isotonic conditions. A larger I_{ClC3} was elicited under the same isotonic conditions. (b) Subsequent hypotonic cell swelling failed to further increase the current amplitude of these cells. (c) Acute application of PDBu (100 nM) under hypotonic conditions no longer inhibited the currents. (d) Mean I–V curves from four different cells under isotonic (○), isotonic BIM 100 nM (●), and hypotonic PDBu 100 nM (■) conditions. (C) Inhibition of endogenous PKC by BIM (100 nM) activated ClC-3 channels in isotonic solutions, and subsequent hypotonic cell swelling failed to further increase the current density. (a) I_{ClC3} under isotonic conditions. (b) Exposure of the same cell to BIM (100 nM) under isotonic condition increased the current. (c) Subsequent exposure of the cell to hypotonic solution caused no further increase in current amplitude. (d) Mean I–V curves from five different cells under isotonic (○), isotonic BIM 100 nM (●), and hypotonic BIM 100 nM (■) conditions.
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-Lys-Asn-Ser51-Lys-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\textsuperscript{-}]\textsubscript{o} was replaced by equimolar (90 mM) I\textsuperscript{-} or Asp\textsuperscript{-} (E\textsubscript{Cl} = +46 mV). I\textsuperscript{-} and Asp\textsuperscript{-} substitution of [Cl\textsuperscript{-}]\textsubscript{o} shifted the reversal potential of I\textsubscript{Cl,vol} in atrial myocytes (C) from −2.0 ± 0.5 to −15.3 ± 1.3 and +39.5 ± 1.7 mV (n = 6), respectively, and of I\textsubscript{Cl,vol} in ventricular myocytes (D) from −3.2 ± 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.

![Figure 2](https://jgp.rupress.org/content/48/1/61/F2.large.jpg)

**Figure 2.** PKC activity and volume-regulation of native I\textsubscript{Cl,vol} in guinea-pig atrial (A) and ventricular (B) myocytes. (a) Representative whole-cell current traces recorded under isotonic conditions. (b) Subsequent exposure of the same cell to hypotonic solutions caused a further increase in the currents (I\textsubscript{Cl,swell}). (c) Activation of PKC by PDBu (100 nM) under hypotonic conditions caused closure of most channels. (d) Mean I-V relationships from different atrial (n = 6, in A) or ventricular (n = 5, in B) cells under isotonic (O), hypotonic (●), and hypotonic PDBu 100 nM (▲) conditions. (C and D) I-V relationship of whole-cell currents in atrial (C) and ventricular (D) myocytes before and after [Cl\textsuperscript{-}]\textsubscript{o} was replaced by equimolar (90 mM) I\textsuperscript{-} or Asp\textsuperscript{-} (E\textsubscript{Cl} = +46 mV). I\textsuperscript{-} and Asp\textsuperscript{-} substitution of [Cl\textsuperscript{-}]\textsubscript{o} shifted the reversal potential of I\textsubscript{Cl,vol} in atrial myocytes (C) from −2.0 ± 0.5 to −15.3 ± 1.3 and +39.5 ± 1.7 mV (n = 6), respectively, and of I\textsubscript{Cl,vol} in ventricular myocytes (D) from −3.2 ± 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.
rent density of 952 ± 41 pA pF⁻¹ at +80 mV and −606 ± 71 pA pF⁻¹ at −80 mV, P = NS). Similar results were obtained from S51ACGC-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 $930 ± 18$ pA pF⁻¹ at +80 mV and $−608 ± 22$ pA pF⁻¹ at −80 mV, $n = 7$) that was no longer responsive to cell swelling (Fig. 5 C, b, mean

Figure 3. Inhibition of endogenous PKC by BIM (100 nM) activated large outwardly rectifying CI⁻ currents under isotonic conditions in guinea-pig atrial (A) and ventricular (B) myocytes. Subsequent hypotonic cell swelling failed to significantly increase the current densities in these cells. (a) Whole-cell current recordings under isotonic conditions. (b) Exposure of the same cell to BIM (100 nM) under isotonic conditions caused a significant increase in the membrane current with similar biophysical properties as $I_{\text{ClC-3}}$ (see A). (c) Subsequent exposure of the cell to hypotonic solution caused no significant increase in current amplitude. (d) Mean I-V curves from six (C and D) different cells under isotonic (○), isotonic BIM 100 nM (●), and hypotonic BIM 100 nM (□) conditions. (C) Pharmacology of BIM-induced $I_{\text{ClC-3}}$ in atrial myocytes. a and b compare the effects of DIDS (100 μM) on the swelling-induced $I_{\text{ClC-3}}$ (a) and the BIM-induced $I_{\text{ClC-3}}$ (b) in atrial myocytes. DIDS inhibited these currents in an identical voltage-dependent manner. Extracellular ATP (10 mM) also inhibited BIM-induced $I_{\text{ClC-3}}$ in a characteristic voltage-dependent manner (c) identical to its inhibitory effect on the wild-type gpClC-3 channels (Duan et al., 1997b) and $I_{\text{ClC-3}}$ in many other tissues and species (Strange et al., 1996).
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 current density of 907 ± 31 pA pF⁻¹ at +80 mV and −627 ± 31 pA pF⁻¹ at −80 mV, P = NS compared with isotonic and hypotonic conditions). As shown in Fig. 5 D, however, the S362AClC-3 mutant yielded a channel with an intermediate responsiveness to cell swelling and PKC. The mean current densities of S362AClC-3 under isotonic conditions were 610 ± 42 pA pF⁻¹ at +80 mV and −348 ± 31 pA pF⁻¹ at −80 mV (n = 6) that are significantly higher (P < 0.05) than the mean current densities of wtClC-3 under the same isotonic conditions. Exposure of these S362AClC-3–transfected cells to hypotonic 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 isotonic conditions). Activation of PKC by PDBu under hypotonic conditions caused significantly less inhibition 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 randomized 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), wtClC-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, respectively. While only very small currents could be detected from CD8-transfected cells under isotonic and hypotonic conditions with similar densities as reported before (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 CIC 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 significant response to changes in cell volume (Fig. 6 B) or activation of PKC (Fig. 6 C). Basal S362AClC-3 currents 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 (ATPₘ, 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 I_Cl.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 I_Cl.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

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.
channels (Fig. 8 C, c and d), confirming the involve-
b, respectively, ionomycin failed to inhibit S51AClC-3
a and b) were identical to those shown in Fig. 5 C, a and
nels under isotonic and hypotonic conditions (Fig. 8 C,
ionomycin. While the properties of S51AClC-3 chan-
the response of expressed mutant S51AClC-3 channel to
b
lular Ca2+
changes to close the channel. To further examine
the ClC-3 protein and cause similar conformational
sensitive and -insensitive PKC isozymes can phosphorylate
is dependent on PKC phosphorylation of the
isozymes (Gekeler et al., 1996; Toul-

Figure 6. Comparison of current densities at +80 mV in NIH/3T3 cells transfected with CD8, wtClC-3, S51AClC-3, S362AClC-3, and S51A+ S362AClC-3 under isotonic (A), hypotonic (B), and hypotonic PDBu 100 nM (C) conditions, respectively. Whole-cell currents were recorded from cells transfected with different cDNAs that were randomly coded during experiments. Results were revealed after all experiments were completed.

The reason for this discrepancy is unclear. Both Ca2+-
sensitive (PKCa) and -insensitive (PKCδ and PKCe)
PKC isozymes are abundantly expressed in NIH/3T3 cells (Szallasi et al., 1994), and BIM inhibits all PKCa, β1, βII, γ, δ, and ε isozymes (Gekeler et al., 1996; Toullec et al., 1991). Thus, it is possible that both Ca2+-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 intracel-
ular Ca2+ 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 involve-
currents by ionomycin was prevented by inhibition of endogenous PKC when cells were pretreated with BIM (100 nM). These results suggest that inhibition of gp-
CIC-3 channels by an increase in intracellular Ca2+ may be due to a Ca2+-sensitive PKC-phosphorylation–mediated mechanism. This is different from rat ClC-3 channelks (Kawasaki et al., 1995) in which the Ca2+ 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 Ca2+-sensitive (PKCa) and -insensitive (PKCδ and PKCe) PKC isozymes are abundantly expressed in NIH/3T3 cells (Szallasi et al., 1994), and BIM inhibits all PKCa, β1, βII, γ, δ, and ε isozymes (Gekeler et al., 1996; Toullec et al., 1991). Thus, it is possible that both Ca2+-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 intracel-
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nment of amino-terminal PKC-phosphorylation in the Ca2+ 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 ICl.vol currents in guinea-pig atrial and ven-
tricular myocytes. Our functional and mutational stud-
ies of the ClC-3 gene product indicate that the activa-
tion of ClC-3 channels during hypotonic-induced cell swelling is attributable to relief of endogenous PKC inhibition of these channels caused by cell swelling–
duced dephosphorylation of a serine residue within the amino terminus of the channel protein. Thus, these results provide an important new clue into the molecu-
lar link between changes in cell volume, protein phospho-
dylation–dephosphorylation, and channel function.

Protein phosphorylation or dephosphorylation is a common rapid and reversible means of transducing sig-
als from the extracellular environment to many cellu-
lar 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 confine-
ment, 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). Alternative,
slowly 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 mes-
senger 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 regula-
tion may be due to alterations in gene expression of second messengers (Waldegger et al., 1997a; Waldeg-
ger and Lang, 1998). The exact interaction between cell volume and elements of intracellular signaling and detailed intermediate processes of how PKC and PP ac-
tivities may be regulated by cell volume requires fur-
ther study.

Our data suggests that ClC-3 channels may exist in ei-
ther a closed phosphorylated state or an active dephos-
phorylated state. PKC phosphorylation of the NH₂ terminus of ClC-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; Coca-Prados et al., 1995, 1996; Strange et al., 1996; Dove et al., 1997; Waldegger et al., 1997a), we propose that ClC-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 ClC-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, 1991; Starke and Jennings, 1993; Palfrey, 1994; Lytle, 1998); ClC-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 ClC-3 mutants in NIH/3T3 cells. (A) Representative whole-cell recording of S51A ClC-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 ClC-3 current, extracellular ATP (10 mM), DIDS (100 µM), and tamoxifen (TMX, 10 µM) all inhibited S51A ClC-3 currents in a characteristic voltage-dependent manner identical to their inhibitory effects on the wtClC-3 channels (Duan et al., 1997b). (B) I–V relationship of whole-cell currents in NIH/3T3 cells transfected with S51A + S362A ClC-3 (a), S51A ClC-3 (b), S362A ClC-3 (c under isotonic conditions and d under hypotonic conditions) before and after [Cl⁻]o was replaced by equimolar (110 mM) I⁻ or Asp⁻ (ECl = +42.8 mV). I⁻ and Asp⁻ substitution of [Cl⁻]o shifted the reversal potential of S51A + S362A ClC-3 current (a) from −1.0 ± 1.2 to −14.2 ± 1.1 and +43.8 ± 3.2 mV (n = 4), respectively, of S51A ClC-3 current (b) from −3.8 ± 0.3 to −14.9 ± 2.6 and +37.7 ± 1.6 mV (n = 3), respectively, of S362A ClC-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 ClC-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.
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 ClC-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 Cl2 conductances in hippocampal pyramidal cells (Madison et al., 1986), resting Cl2 conductance in vascular smooth muscle cells (Sagusa and Kokubun, 1988), skeletal muscle cells (Brinkmeier and Jockusch, 1987), ClC-1 in skeletal muscle and HEK cell expression system (Rosenbohm et al., 1995), and the hyperpolarization-activated ClC-2 Cl2 channel that is also regulated by cell volume (Staley, 1994; Staley et al., 1996; Fritsch and Edelman, 1996, 1997). Consistent with our finding in native guinea-pig atrial and ventricular myocytes and cloned ClC-3–transfected NIH/3T3 cells, PKC activation inhibits ICl.vol and outwardly rectifying chloride channel in rabbit atrial myocytes (Duan et al., 1995), MDR1-transfected 3T3

![Figure 8](image_url)

**Figure 8.** Effects of increase in intracellular Ca2+ on wild-type and mutant S51A ClC-3 currents in NIH/3T3 cells. (A) Ionomycin (1 μM) inhibited wtClC-3 currents under hypotonic conditions in the presence of 2.5 mM external Ca2+. Representative whole-cell currents recorded under isotonic, hypotonic, and hypotonic ionomycin conditions and the mean I–V curves of each (n = 4) are shown in a–d, respectively. (B) Pretreatment of wtClC-3 transfected NIH/3T3 cell with BIM (100 nM) under isotonic condition (a) abolished the upregulation effect by hypertonic 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 ClC-3 (S51A ClC-3) also abolished the upregulation effect by hypertonic 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_{Cl,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 upregulated I_{Cl,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_{Cl,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_{Cl,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; Szűcs 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_{Cl,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 isozymes 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 isozymes. 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_{Cl,vol}. Simple biochemical experiments or attempts to measure activities or translocations of PKC or PKC isozymes 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 alternations 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/ClC-4/ClC-5 subbranch (Jentsch, 1996) are also involved in cell-volume regulation and account for I_{Cl,vol} in other cell types needs further investigation.

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