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Swelling-Induced Chloride Currents in Neuroblastoma Cells Are Calcium Dependent

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The effects of osmotic stress on chloride (Cl^-) currents in the human neuroblastoma cell line CHP-100 were evaluated. Following exposure to hypoosmotic solution, an increase in whole-cell Cl^- current was observed. This current was blocked by the Cl^- channel blocker 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB). In cells loaded with the Cl^- permeability marker ^{125}I, exposure to hypoosmotic solution increased ^{125}I efflux by 197 ± 14% ($n = 41$, $p < 0.05$) over controls. This increase was sensitive to NPPB. Hypoosmotic stress also increased cytosolic calcium levels (Ca^{2+}) in fura-2–loaded cells. Pretreatment with EGTA inhibited the increase in cytosolic Ca^{2+}, ^{125}I efflux, and whole-cell Cl^- current produced by hypoosmotic solution. Antagonists of N-, L-, and T-type Ca^{2+} channels did not alter stimulation in ^{125}I efflux or cytosolic Ca^{2+} levels during osmotic stress. However, ω-conotoxin MVIIC, a P-type Ca^{2+} channel blocker, inhibited hypoosmotically activated whole-cell Cl^- currents and increases in cytosolic Ca^{2+}. It is concluded that a Ca^{2+}-dependent change in Cl^- permeability is activated in CHP-100 cells in response to osmotic stress.

[Key words: hypoosmotic, chloride currents, calcium-activated conductance, nystatin perforated patch, calcium channels]

Most cells possess a variety of mechanisms for regulatory volume decrease (RVD) in response to osmotic swelling. One common mechanism is activation of plasma membrane potassium (K^+) and/or Cl^- channels that leads to loss of K^+, Cl^- and/or amino acids and the subsequent loss of water (Hoffmann and Simonsen, 1989; Parker, 1993). Swelling-induced activation of ion permeabilities has been demonstrated in some cells of the nervous system, including rat astrocytes (O'Connor and Kimelberg, 1993; Pasantes-Morales et al., 1994) and neuroblastoma N1E115 cells (Falke and Misler, 1989).

Several intracellular second messenger systems including cAMP, protein kinases, phosphatidylinositols, and Ca^{2+} may regulate ion permeability changes activated during osmotic insult (Hoffman and Simonsen, 1989; Sarkadi and Parker, 1991). The effects of Ca^{2+} on volume-activated Cl^- permeability are dependent upon the cell type studied. In rat epididymal cells (Chan et al., 1993), lymphocytes (Grinstein et al., 1984), Ehrlich ascite cells (Hoffman et al., 1984), and human intestinal 407 cells (Kubo and Okada, 1992), extracellular Ca^{2+} does not influence volume-activated Cl^- channels, but release of intracellular Ca^{2+} may play an important role. In contrast, in MDCK cells (Rothstein and Mack, 1990), eccrine clear cells (Ohtsuyama et al., 1993), and other cell types (McCarty and O'Neil, 1992), influx of Ca^{2+} stimulates a volume-activated Cl^- permeability leading to RVD. Conflicting information regarding the role of extracellular calcium has been reported in astrocytes. Pasantes-Morales et al. (1994) suggest RVD is independent of Ca^{2+} influx, while O'Connor and Kimelberg (1993) report that extracellular Ca^{2+} is necessary for RVD.

The present study evaluates the role of extracellular Ca^{2+} on Cl^- permeability changes following osmotic stress in the human neuroblastoma cell line CHP-100. Using patch-clamp recordings, radioisotopic efflux, and Ca^{2+} fluorescence measurements, we have characterized a swelling-induced Cl^- conductance that is dependent upon an influx of Ca^{2+}.

Materials and Methods

Cell culture. The human neuroblastoma cell line CHP-100 was kindly provided by Dr. Audrey Wilson and Mr. Al Wilson from the Children's Hospital of Philadelphia. Cells were maintained in culture at 37°C in a 5% CO_2 incubator in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Isotope efflux. Using previously described techniques (Venglarik et al., 1990; Basavappa et al., 1993), ^{125}I (Dupont) efflux was used to assess Cl^- permeability in cells grown to 75–90% confluence on 22 mm plates (Costar). Cells were incubated with extracellular buffer (see below) containing 10 μCi/ml ^{125}I at room temperature. After 1 hr, cells were washed three times with normal extracellular buffer to remove extracellular isotope. Studies were initiated by adding and removing 1 ml of extracellular buffer at 30 sec intervals. Cells were exposed to the potassium channel blocker 4-aminopyridine (3 mM) during the course of the entire experiment to eliminate a contribution of potassium permeability to the osmotic response. After a 2 min period to establish basal efflux, hypotonic buffer was added. Cells were pretreated with test agents for 5 min prior to hypoosmotic solution and during exposure to hypoosmotic stress. At the end of the study, cells were lysed with 1 ml of 0.1*N* NaOH to determine the total amount of radioactivity remaining. Results were normalized by dividing counts at each time point by the total number of counts remaining. Data are presented as percentage change in peak ^{125}I efflux, to examine the effects of test agents on hypoosmotically stimulated efflux.

Cytosolic Ca^{2+} measurements. Intracellular Ca^{2+} concentration was measured using the Ca^{2+}-sensitive fluorochrome fura-2 (AM) (Molecular Probes, Eugene, OR). Cells in suspension were loaded for 30 min with 20 μM fura-2 in Dulbecco's Modified Eagle's Medium (DMEM) (Bio Whittaker, Walkersville, MD). Cells were then washed twice with

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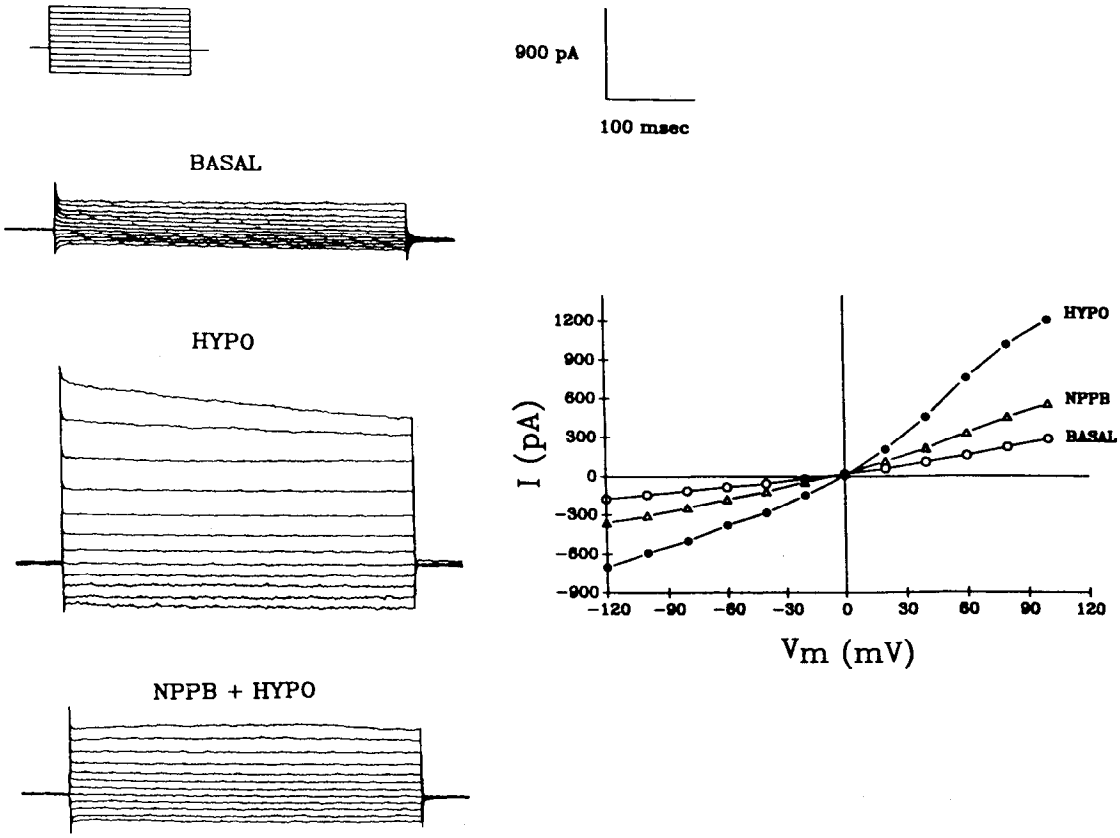

Figure 1. Osmotic stress activates Cl^- currents. In this representative nystatin perforated-patch recording, the standard extracellular solution was supplemented with 5 mM $BaCl_2$ to block K^+ currents. Membrane voltage was held at −40 mV and stepped to test potentials between −120 and 100 mV in 20 mV increments (400 msec in duration) (see *inset*). Membrane currents (*left*) and current–voltage relationship (*right*) are illustrated under basal conditions (*open circles*), after exposure to hypoosmotic solution (*closed circles*), and subsequently, after 15 μM NPPB (*open triangles*). NPPB inhibited the hypoosmotically activated currents.

PBS and transferred to a cuvette containing Hank's balanced salt solution containing 10 mM HEPES (pH 7.4) and placed in a Perkin Elmer LS50B spectrometer. Real time measurements were monitored by alternating excitation wavelengths (340/380 nm) every 1.9 sec with a microcomputer, and data were derived from the ratio of emissions at 510 nm. F_{max} and F_{min} were determined with Triton X-100 and EGTA, respectively. Intracellular Ca^{2+} was calculated as described by Grynkiewicz et al. (1985).

Patch-clamp studies. The nystatin perforated patch-clamp recording technique was used for measurement of whole-cell currents (Horn and Marty, 1988). Immediately before study, medium in the culture dish was replaced with extracellular buffer (see below) containing either 5 mM $BaCl_2$ or 3 mM 4-AP to block K^+ currents. Cells were viewed at 600× magnification with Hoffman optics using an inverted phase contrast microscope (Olympus IMT-2). Recording pipettes were pulled from Corning 7052 glass and had a resistance of 3–5 MΩ when filled with intracellular buffer (see below). Recordings were made with an Axopatch 1D amplifier (Axon Instruments). Voltage and current commands were performed using pCLAMP programs (Axon Instruments), a Digidata 1200 interface (Axon Instruments), and a Compaq 486 DX computer. For whole-cell studies, membrane potential was held at −40 mV, and current responses measured during 20 mV (400 msec duration) voltage steps between −120 mV and 100 mV.

Solutions and reagents. The extracellular buffer contained (in mM) 140 NaCl, 4 KCl, 1 KH_2PO_4, 2 $MgCl_2$, 1.5 $CaCl_2$, 3 glucose, and 10 HEPES (pH 7.4 with NaOH) (290 mOsm/Kg H_2O by an osmometer). The final hypoosmotic solution contained (in mM) 93 NaCl, 4 KCl, 1 KH_2PO_4, 2 $MgCl_2$, 1.5 $CaCl_2$, 3 glucose, and 10 HEPES (pH 7.4 with NaOH) (190 mOsm/kg H_2O by an osmometer). The intracellular pipette solution contained (in mM) 10 NaCl, 130 KCl, 2 $MgCl_2$, and 10 HEPES (pH 7.4 with KOH).

NPPB (Bimol Research Labs Inc., Plymouth Meeting, PA) was dissolved in DMSO with a final bath concentration of <1% DMSO. An equal amount of DMSO had no effect on membrane currents or ^{125}I efflux. ω-Conotoxin MVIIC was obtained from Peptides International (Louisville, KY), and thapsigargin, from Calbiochem (La Jolla, CA). All other agents were obtained from Sigma Chemical Co. (St. Louis, MO). All experiments were performed at room temperature.

Data analysis. Results are presented as mean ± SE, where n refers to number of cells for electrophysiologic recording, monolayers for efflux studies, and cuvettes of cells for Ca^{2+} fluorescence. Statistical comparisons were by Student's t test with a significance level of $p < 0.05$.

Results

Activation of Cl^- currents by hypoosmotic solution. In isoosmotic solution, CHP-100 cells showed only low level basal currents (Fig. 1). Exposure to hypoosmotic solution activated whole-cell currents within 2 min (Fig. 1). In 16 of 16 cells a similar response was observed. The whole-cell currents stimulated by hypoosmotic solution showed a mean reversal potential of −16 ± 3 mV and peak currents increased by 220 ± 21 pA, 322 ± 38 pA, 422 ± 53 pA, and 527 ± 62 pA at 40, 60, 80, and 100 mV, respectively. Addition of the Cl^- channel blocker NPPB (15 μM) (Kunzelmann et al., 1992) inhibited the hypoosmotic-induced current in five of five cells tested (Fig. 1).

The effects of hypoosmotic solution on anion permeability were further evaluated by measuring ^{125}I efflux, a marker for chloride permeability. Exposure to hypoosmotic solution increased peak efflux by 197 ± 14% ($n = 41$, $p < 0.05$) over basal levels (Fig. 2). Stimulation of ^{125}I efflux began within 1 min of addition of hypoosmotic solution, reaching a maximal

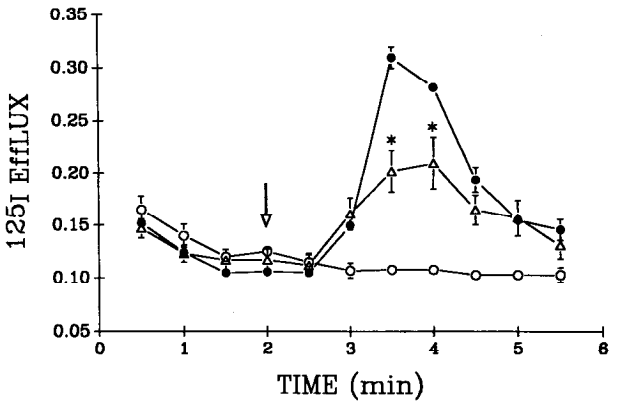

Figure 2. Effects of hypoosmotic stress on ^{125}I efflux. In this representative study, CHP-100 cells under control conditions (*open circles*, $n = 3$) showed a gradual decline in efflux. Addition of hypoosmotic solution (at *arrow*) caused an increase in ^{125}I efflux within 1 min of exposure (*closed circles*, $n = 3$). Cells pretreated with NPPB (15 μM) for 5 min prior to hypoosmotic solution, and during exposure to hypoosmotic stress, showed an inhibition of ^{125}I stimulated efflux (*open triangles*, $n = 6$). The bath solution contained 3 mM 4-AP to block K^+ currents. Error bars (SE) are not evident when they are smaller than the size of the symbol. *, $p < 0.05$ with respect to hypoosmotic solution.

stimulation after 1.5–2 min. This increase was followed by a gradual decline towards basal levels over the next 2 min (Fig. 2). In cells treated with NPPB (15 μM; $n = 10$), a significant ($p < 0.05$) inhibition of the stimulation in ^{125}I efflux by hypoosmotic solution was observed (Fig. 2, Table 1).

Ca^{2+} dependence of Cl^- currents. In many cell types, RVD activated currents are Ca^{2+} dependent (for review, see McCarty and O'Neil, 1992). To determine if increases in cytosolic Ca^{2+} occur in CHP-100 cells after osmotic stress, cells were loaded with the Ca^{2+}-sensitive fluorochrome fura-2 (AM) (20 μM). Hypoosmotic solution increased cytosolic Ca^{2+} from a basal level of 58 ± 7 nM ($n = 24$) to a peak level of 150 ± 14 nM Ca^{2+} ($n = 7$, $p < 0.05$) (Fig. 3, Table 1). Characteristically, this increase was rapid, reaching a peak within 60 sec, and declining over the next 180 sec. However, a persistent tail was often noted for over 400 sec.

To determine if increases in cytosolic Ca^{2+} may serve as a signal for activation of Cl^- permeability, whole-cell currents and ^{125}I efflux measurements were made in cells exposed to hypoosmotic solution containing 2.5 mM EGTA. Under these conditions, hypoosmotic solution failed to significantly ($p > 0.05$) increase whole-cell currents ($n = 4$) (Fig. 4). Peak ^{125}I efflux was significantly ($p < 0.05$) reduced following exposure to hypoosmotic solution containing EGTA. In the absence of extracellular Ca^{2+} (i.e., EGTA-containing solution) hypoosmotic solution increased efflux by only 19 ± 5% ($n = 27$; Table 1) of the hypoosmotic-induced response seen in the presence of Ca^{2+}. Pretreatment of CHP-100 cells with 2.5 mM EGTA reduced basal cytosolic Ca^{2+} levels to 37 ± 7 nM ($n = 5$), and significantly ($p < 0.05$) reduced the hypoosmotically induced elevation in cytosolic Ca^{2+} to 64 ± 7 nM ($n = 5$; Table 1).

Ca^{2+} entry pathway. Inhibition of hypoosmotically induced whole-cell currents and ^{125}I efflux by removal of extracellular Ca^{2+} with EGTA suggest that influx of Ca^{2+} is, at least partially, responsible for the increase in cytosolic Ca^{2+} that activates Cl^- currents in CHP-100 cells. To evaluate the specific Ca^{2+} entry pathway involved, cells were pretreated for 5 min, and exposed for the duration of the study, with specific blockers of N-, L-, T-, and P-type Ca^{2+} channels. ω-Conotoxin GVIA is a specific N-type Ca^{2+} channel blocker, and these channels have previously been demonstrated in CHP-100 cells (Basavappa et al., 1994). Exposure to ω-conotoxin GIVA (2 μM) did not significantly affect the peak hypoosmotically stimulated ^{125}I efflux (99 ± 7% of the hypoosmotic response; $n = 12$) (Table 1). Similar lack of inhibition of hypoosmotically induced ^{125}I efflux was observed with the L-type Ca^{2+} channel blocker diltiazem (10 μM) (95 ± 9%; $n = 12$) (see Table 1) and the T-type blocker nickel (1 mM) (101 ± 6%; $n = 12$) (Table 1). Neither ω-conotoxin GIVA nor diltiazem significantly ($p > 0.05$) altered the elevation in cytosolic Ca^{2+} seen after exposure to hypoosmotic solution (Table 1). In contrast, ω-conotoxin MVIIC (10 μM), a P-type Ca^{2+} channel blocker (Hillyard et al., 1992), inhibited hypoosmotically activated Cl^- currents ($n = 4$; Fig. 5) and significantly reduced the increase in cytosolic Ca^{2+} produced by exposure to

Table 1. ^{125}I efflux and cytosolic Ca^{2+} levels in CHP-100 cells following hypoosmotic stress

	^{125}I efflux (%)	n	Cytosolic Ca^{2+} (nM)	n
Basal	—	—	58 ± 7	24
Hypoosmotic	100	41	150 ± 14*	7
NPPB (15 μM)	46 ± 7*	10	—	
EGTA (2.5 mM)	19 ± 5*	27	64 ± 6	5
ω-Conotoxin GVIA (2 μM)	99 ± 7	12	119 ± 17*	4
Diltiazem (10 μM)	95 ± 9	12	128 ± 20*	4
Nickel (1 mM)	101 ± 6	12	—	
ω-Conotoxin MVIIC (10 μM)	—		72 ± 5	4

Cells were treated with test agents prior to and during exposure to hypoosmotic solution. Results for efflux studies are presented as a percentage of the peak hypoosmotically stimulated ^{125}I efflux corrected for basal efflux. For Ca^{2+} fluorescence experiments, peak increases in Ca^{2+} after test treatments are compared to basal calcium levels.

* Significantly ($p < 0.05$) different from hypoosmotically stimulated efflux or basal Ca^{2+} levels.

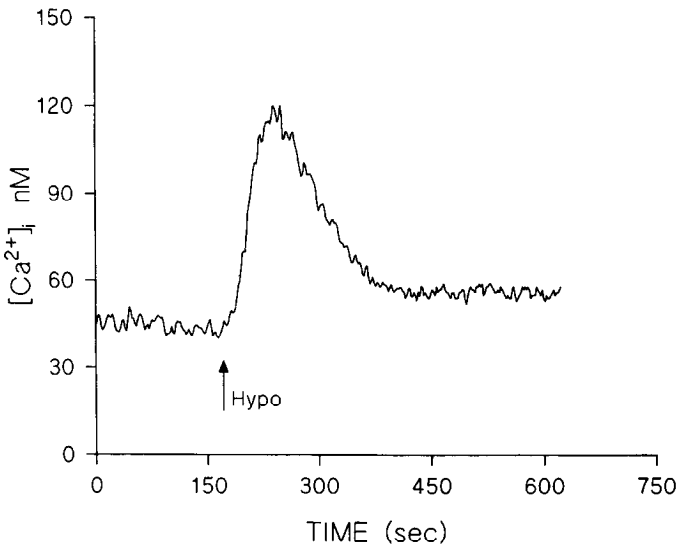

Figure 3. Increase in cytosolic Ca^{2+} in response to osmotic stress. Cells were loaded for 30 min with 20 μM fura-2 (AM) and cytosolic Ca^{2+} measured as described in Materials and Methods. Addition of hypoosmotic solution (at *arrow*) rapidly increased the cytosolic Ca^{2+} level.

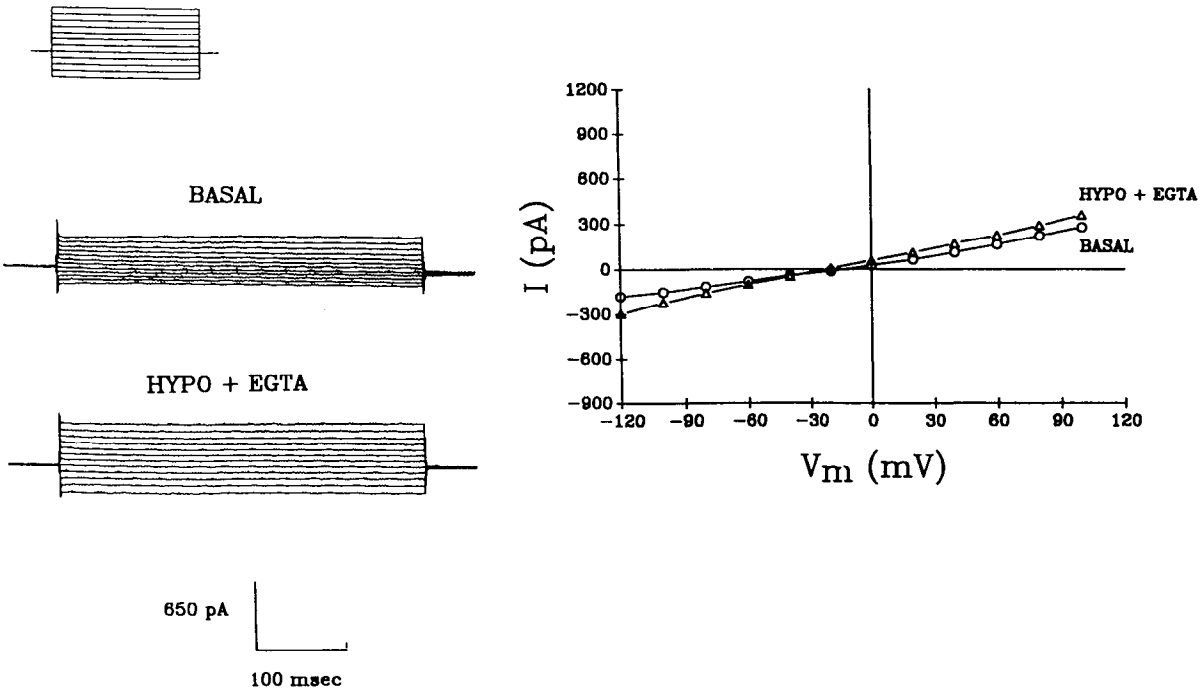

Figure 4. Effects of EGTA on whole-cell Cl^- currents. Membrane currents (*left*) and current–voltage relationship (*right*) are shown under basal conditions, and following addition of hypoosmotic solution to cells pretreated with 2.5 mM EGTA for 5 min. Hypoosmotically activated currents were markedly decreased as compared to currents in the absence of EGTA (see Fig. 1).

hypoosmotic solution to 72 ± 5 nM (Table 1), without affecting basal Ca^{2+} levels.

To confirm that the inhibition in Cl^- current by ω-conotoxin MVIIC was secondary to blockade of Ca^{2+} influx, the Ca^{2+}-ATPase inhibitor thapsigargin (10 μM) was added after exposure to hypoosmotic solution containing ω-conotoxin MVIIC. Both whole-cell Cl^- current and cytosolic Ca^{2+} levels increased after thapsigargin, suggesting that the Ca^{2+}-activated Cl^- cur-

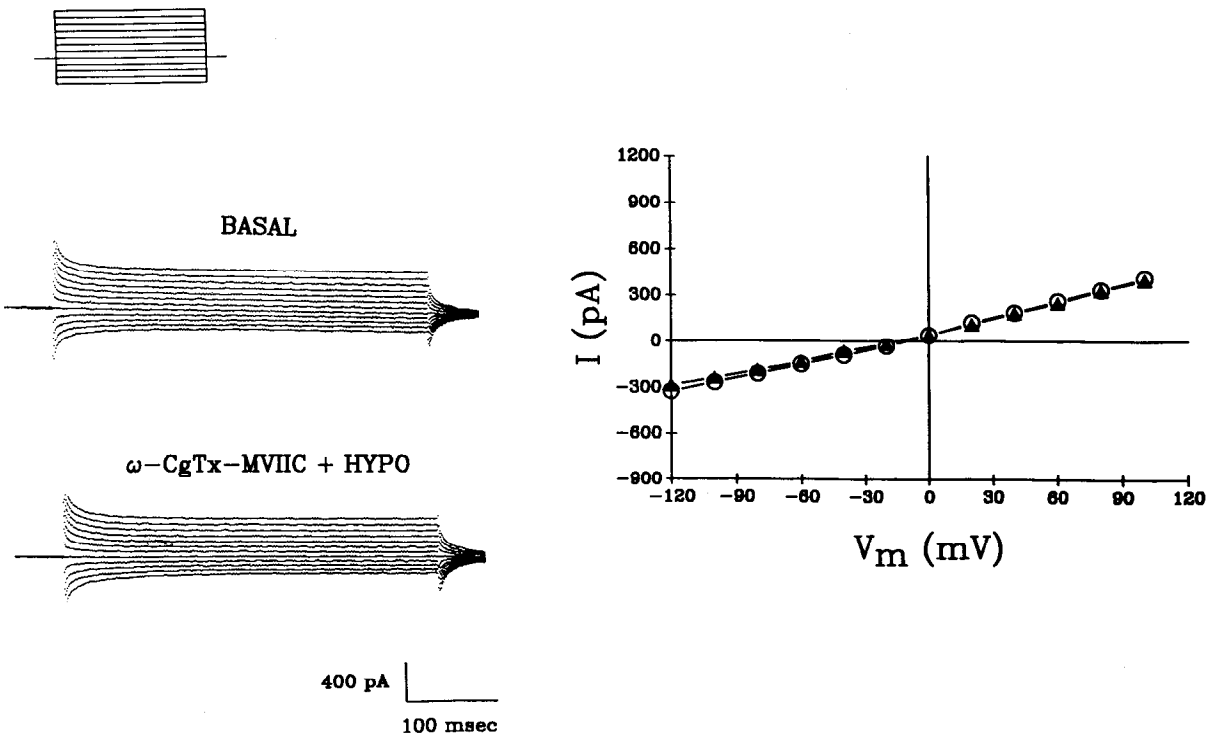

Figure 5. Effects of ω-conotoxin MVIIC on whole-cell currents. Membrane currents (*left*) are shown under basal conditions and following hypoosmotic stress in cells pretreated with 10 μM ω-conotoxin MVIIC for 5 min. The *right panel* shows the corresponding current–voltage relationship. *Open circles,* basal currents; *closed triangles*, hypoosmotic response after treatment with ω-conotoxin MVIIC. ω-Conotoxin MVIIC markedly inhibited the stimulation in Cl^- current by hypoosmotic solution.

rent "machinery" was not inhibited by ω-conotoxin MVIIC, per se. The effects of ω-conotoxin MVIIC could not be examined on ^{125}I efflux as quantities required for efflux studies are prohibitive.

Discussion

Exposure to hypoosmotic solution activates a current in CHP-100 cells. Several lines of evidence indicate that this current is carried by Cl^-. (1) Whole-cell reversal potential approached E_{Cl}, and currents were markedly reduced by the Cl^- channel blocker NPPB. (2) ^{125}I efflux, a marker for Cl^- permeability, was increased following exposure to hypoosmotic solution and this response was inhibited by NPPB. (3) The contribution of K^+ channels was removed by the addition of K^+ channel blockers.

Cytosolic Ca^{2+} levels increased in CHP-100 cells during exposure to hypoosmotic solution. This increase was sensitive to removal of extracellular Ca^{2+} with EGTA, a maneuver that decreased stimulation of both whole-cell Cl^- currents and ^{125}I efflux. Ca^{2+} influx during osmotic stress does not appear to use N-, L-, or T-type Ca^{2+} channels, since specific blockers of these channel types did not significantly effect ^{125}I efflux or cytosolic Ca^{2+} measurements. In contrast, ω-conotoxin MVIIC inhibited both the increase in cytosolic Ca^{2+} and activation of Cl^- currents after osmotic stress. Initial studies with ω-conotoxin MVIIC demonstrated inhibition of P-type channels in cerebellar Purkinje cells and CA1 hippocampal pyramidal cells (Hillgard et al., 1992). However, recent evidence (Wheeler et al., 1994; Zhang et al., 1994) suggests Q- and R-type Ca^{2+} channels may also be affected. Thus, ω-conotoxin MVIIC–sensitive Ca^{2+} channels are activated in CHP-100 cells as a consequence of osmotic stress, but the exact identity of the channel subtype requires additional investigation.

During hypoosmotic stress, Cl^- conductance is activated in many cell types (Kubo and Okada, 1992; Valverde et al., 1992; Chan et al., 1993; Zhang et al., 1993; Kelly et al., 1994). These Cl^- currents show differing dependence upon extracellular Ca^{2+} (for review, see McCarty and O'Neil, 1992). For instance, in astrocytes hypoosmotically activated RVD and Cl^- efflux are inhibited in the absence of Ca^{2+} (O'Connor and Kimelberg, 1993). These studies suggest that the Ca^{2+} influx pathway is through an L-type Ca^{2+} channel, as the selective channel blocker nimodipine produces an identical effect. In contrast, Pasantes-Morales et al. (1994), also in studies with astrocytes, found RVD to be independent of extracellular Ca^{2+}.

In summary, in CHP-100 cells, osmotic stress may lead to activation of ω-conotoxin MVIIC–sensitive Ca^{2+} channels. The resulting increase in cytosolic Ca^{2+} activates Cl^- channels, which may lead to a loss of Cl^- and H_2O, with subsequent decrease in cell size. The present study represents the first example describing a role for P-, and/or Q- or R-type Ca^{2+} channels in activation of Cl^- channels after osmotic stress. CHP-100 cells may represent a useful model for evaluating RVD in the nervous system.

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