Pflügers Arch – Eur J Physiol (1997) 435:9–19

ORIGINAL ARTICLE

Wen Xie Xu · Sung Joon Kim · Insuk So
Tong Mook Kang · Jong Chul Rhee · Ki Whan Kim

Volume-sensitive chloride current activated by hyposmotic swelling in antral gastric myocytes of the guinea-pig

Received: 6 June 1997 / Received after revision and accepted: 24 July 1997

Abstract The characteristics of volume-sensitive chloride current (I_{Cl}) induced by osmotic cell swelling were studied using the whole-cell patch-clamp technique and cell diameters of antral circular guinea-pig myocytes were simultaneously measured under isosmotic and hyposmotic conditions by using a video image analysis system. At –60 mV, osmotic cell swelling (200 mosmol/l) activated a sustained inward current. Instantaneous current/voltage (*I-V*) relations obtained by step voltage pulses showed an outward rectification. At potentials above +40 mV, the current exhibited time-dependent decay. The outward current amplitude was decreased and the reversal potential was shifted to more positive potentials by replacement of external Cl^- with $gluconate^-$, while the current amplitude and the *I/V* relation were not affected by replacing extracellular Na^+ with *N*-methyl-D-glucamine. The anion permeability sequence of the swelling-induced current was I^- (1.80) > Br^- (1.31) > Cl^- (1) > F^- (0.85) > $gluconate^-$ (0.18). The I_{Cl} was effectively inhibited by the Cl^- channel blockers, 4,4′-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS, 100 μM), and niflumic acid (10 μM). DIDS suppressed outward current more effectively than inward current. Also, the I_{Cl} was dose-dependently inhibited by arachidonic acid, an unsaturated fatty acid and also inhibited by other unsaturated fatty acids (linoleic acid and oleic acid) but not by stearic acid, a saturated fatty acid. The inhibitory effect of arachidonic acid on I_{Cl} was not prevented by indomethacin, a cyclo-oxygenase inhibitor and chelerythrine, a protein kinase C inhibitor. Under whole-cell patch-clamp conditions, the cell diameter was continuously measured using video image analysis, which reflects the change in cell volume. A hyposmotic-stimulation-induced increase of cell diameter was followed by I_{Cl} activation. In intact single gastric myocytes, relatively severe hyposmotic (176 mosmol/l) superfusing solution increased the cell diameter and the pretreatment with DIDS or with niflumic acid significantly potentiated the above effect of hyposmotic superfusion. These results suggest that volume-sensitive outwardly rectifying chloride current (I_{Cl}) is present in guinea-pig gastric myocyte and the I_{Cl} may play a role in smooth muscle cell volume regulation.

Key words Smooth muscle · Cell volume · Chloride current

W.X. Xu · S.J. Kim · I. So · K.W. Kim (✉)
Department of Physiology and Biophysics,
Seoul National University College of Medicine,
28 Yongon-Dong, Chongno-Gu, Seoul 110-799, Korea

T.M. Kang
Samsung Biomedical Research Institute, Seoul, Korea

J.C. Rhee
Department of Internal Medicine, College of Medicine,
Sung Kyun Kwan University, Seoul, Korea

Introduction

Cl^- channels have many functions, including the transcellular transport of ions and fluid, cell volume regulation and stabilization of the membrane potential [31, 38]. Recently, a Cl^- channel current activated by hyposmotic cell swelling (I_{Cl}) was reported to be present in various kinds of cells including human epididymal cells [8], human epithelial cells [27], renal cortical collecting duct cell lines [45], rabbit cardiac myocytes [15], rat osteoblast-like cells [12] and rabbit osteoclasts [25]. Activation of K^+ and/or Cl^- currents upon hypotonically induced cell swelling has been reported to occur in a variety of cell species and their role in the regulatory volume decrease (RVD) that takes place after initial cell swelling has been proposed (for reviews see [14, 18, 38]).

Although a hyposmotic-solution-induced Cl^- current in cultured vascular myocytes has been described [20], the physiological role of I_{Cl} in freshly isolated smooth muscle cells and its relationship with volume regulation have not been studied. The Ca^{2+}-activated Cl^- current has been recorded from smooth muscle cells from various tissues [1, 3, 10, 27, 35, 40, 42].

Cells utilize a number of volume regulatory mechanisms to cope with the osmotic imbalance across the cell

membrane: ion channels, exchangers and cotransporters [29]. Roles for the $Na^+/K^+/2Cl^-$ cotransporter and the Na^+/H^+ exchanger in the regulatory volume changes in vascular smooth muscle cells have been reported [40]. However, the role of ion channels in regulatory volume decreases (RVD) or in regulatory volume increases (RVI) in smooth muscle cells has not been studied, especially in relation to gastrointestinal myocytes.

In our previous study, we reported that the voltage-operated calcium current (I_{Ca}) was increased by an osmotically induced membrane stretch [49], and during these experiments we found that a relatively rapid increase of calcium current was followed by a slowly activating inward current (unpublished observation). In this study, we have studied the characteristics of the hyposmotic-stimulation-activated inward current and the experimental evidence suggests that this inward current is carried by Cl^-. Also, we simultaneously measured the cell diameter to examine the physiological role of the chloride current in the regulation of the cell volume of smooth muscle cells.

Materials and methods

Preparation of cells

Guinea-pigs of either sex weighing 300–350 g were exsanguinated after being stunned. The antral part of the stomach was cut and the mucosal layer was separated from the muscle layers in Ca^{2+}-free physiological salt solution (Ca^{2+}-free PSS). The circular muscle layer was dissected from the longitudinal layer using fine scissors and cut into small segments (2 mm × 3 mm). These segments were incubated in a medium modified from the Kraft-Brühe (K-B) medium [24] for 30 min at 4°C. Then they were incubated for 15–25 min at 35°C in digestion medium (Ca^{2+}-free PSS) containing 0.1% collagenase (Wako), 0.05% dithioerythreitol, 0.1% trypsin inhibitor and 0.2% bovine serum albumin. After digestion, the supernatant was discarded and the softened muscle segments were transferred again into the modified K-B medium and single cells were dispersed by gentle agitation with a wide-bore glass pipette. Isolated gastric myocytes were kept in the modified K-B medium at 4°C until use.

Electrophysiological recording

Isolated cells were transferred to a small chamber (0.1 ml) on the stage of an inverted microscope (IMT-2, Olympus, Japan) and continuously superfused with isosmotic PSS by gravity (2–3 ml/min).

Experiments were performed at room temperature (20–25°C) using the whole-cell configuration of the patch-clamp technique [16]. Patch-clamp pipettes were manufactured from borosilicate glass (GC 150T-7.5, Clark Electromedical, UK) using a two-stage puller (PP-83, Narishige, Japan), and fire-polished to give final resistances of 2–4 MΩ when filled with pipette solution. Whole-cell currents were recorded with an Axopatch-1C patch-clamp amplifier (Axon Instruments, USA) and command pulses were applied by using an IBM-compatible 486-grade computer and pClamp software v.5.5.1. The data were displayed on a digital oscilloscope (PM 3350, Philips, The Netherlands), and a computer monitor. The Ag/AgCl reference electrode was connected to the bath solution by a 3 M KCl agar bridge. Junction potentials were measured by changing the external solution from the same pipette solution to normal sodium chloride solution ($[Cl^-]_o$ = 86 mM, see Drugs and solutions) and to solutions of lower $[Cl^-]_o$ (46, 26 and 16 mM). The mean junction potential changes were –0.4 ± 0.2, 0.2 ± 0.2, 0.8 ± 0.4 and 1.2 ± 0.4 mV, respectively, and the observed reversal potentials of I_{Cl} were corrected by these values.

Measurement of single-cell diameter

The diameter of a single myocyte was measured under perforated whole-cell voltage-clamp conditions. The microscope used in this experiment was a Diaphot TMD (Nikon, Japan). Transmitted light was reflected by a full mirror and an image of the cell was projected onto a charge-coupled device (CCD) camera (C 3077, Hamamatsu, Japan), and was then stored using a video tape recorder. For each image, the video camera provided two interleaved frames (odd and even) at a rate of 25/s. During the experiment, each frame was successively digitized and transiently stored on a computer (486-grade IBM-compatible PC) using an 8-bit video digitizer and frame memory system (Cyclope-Digital Vision, France). With the aid of “Digital Vision 4.0” software (Cyclope-Digital Vision), a captured image was used for cell diameter measurement.

Data analysis

Volume-sensitive current and its current/voltage (*I/V*) relation were obtained by digital subtraction of control currents from currents obtained under hyposmotic conditions. The 50% inhibitory concentration (IC_{50}) values for arachidonic acid were calculated by fitting the concentration/inhibition curves to the following logistic function using MicroCal Origin (MicroCal, Northampton, USA).

Percentage of inhibition (%) = $100\{1–1/[\ 1+(x/IC_{50})^n]\}$.

Data are expressed as the mean ± SEM (*n*, number of observations). Statistical significance was tested by Student’s *t*-test and values of $P<0.05$ were considered to be significant.

Drugs and solutions

All drugs used in this experiment were purchased from Sigma (USA). Indomethacin was dissolved in 5% $NaHCO_3$. Niflumic acid and arachidonic acid were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 10 mM. The stock solutions of unsaturated fatty acids were tightly sealed in ampoules and stored at –20°C until use. The vehicle alone had no effect on whole-cell Cl^- currents at the concentrations used (0.1%, n = 6; 0.25%, n = 8) and was always added to control solutions. 4,4’-Diisothiocyanatostilbene-2,2’-disulphonic acid (DIDS) was dissolved in distilled water at 10 mM and added into the test solution.

Ca^{2+}-free PSS containing (mM) NaCl 131, KCl 4.5, glucose 5, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (HEPES) 10 was adjusted to pH 7.4 with tris(hydroxymethyl)aminomethane (TRIZMA). Modified K-B solution containing (mM) L-glutamate 50, KCl 50, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, HEPES 10, ethyleneglycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) 10 was adjusted to pH 7.4 with KOH. External sodium chloride solutions containing (mM) NaCl 80, HEPES 10, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10 were adjusted to 290 and 176–212 mosmol/l with sucrose and pH was adjusted to 7.4 with TRIZMA. NaCl was replaced by the same concentration of *N*-methyl-D-glucamine chloride (NMDG-chloride) in external NMDG chloride solution. For external solutions containing different concentration of chloride, the concentration of NaCl was reduced from 86 mM to 46, 26 and 16 mM, by replacement with sodium gluconate. In all experimental solutions for external perfusion, nicardipine (5 μM) was added to exclude interference from calcium current. The osmolarity of control and hyposmotic solutions was measured using a freezing-point depression osmometer (Advanced Digimatic Osmometer, Model 3D2, USA). Pipette solution containing (mM) CsCl 110, tetraethylammonium (TEA) 20, EGTA 10, HEPES 10, Na_2ATP 3, $MgCl_2$ 3.5 was adjusted to pH 7.3 with TRIZMA.

Results

Outwardly rectifying chloride current activated by hyposmotic stimulation

Under the whole-cell configuration with caesium chloride solution in the pipette (for composition see Materials and methods), the membrane potential was clamped at –60 mV. Applying hyposmotic NaCl solution (200 mosmol/l) slowly activated a sustained inward current as shown in Fig. 1A. The change in cell volume was obvious through the microscope, as shown in Fig. 6, and was followed by activation of an inward current. The discernible activation of inward current occurred about 3 min (3.4 ± 0.24 min, n = 6) after the exchange of bath solution. The inward current and increased cell diameter were reversed slowly upon returning to the isosmotic solution (290 mosmol/l), indicating that the current was volume dependent. Upon returning the cell to the isosmotic solution, a transient increase of inward current was frequently observed (Fig. 1A). The activation of inward current variably reached its steady-state about 4 min (3.6 ± 0.4 min, n = 8) after the discernible change of holding current. As sustained hyposmotic stress over 8 min seemed to be hazardous to the recovery of ionic current and to the repetitive activation of swelling-activated inward current, we usually confined the hyposmotic perfusion to less than 6 min.

Applying hyposmotic NMDG chloride (200 mosmol/l) solution activated an inward current similar to that induced by hyposmotic NaCl solution (Fig. 1A). Step voltage command pulses in the range of –140 to +80 mV produced instantaneous activation of inward and outward currents when the cell was exposed to a hyposmotic NaCl or NMDG chloride solution. Test pulses were preceded by a common depolarizing pulse (0 mV) to confirm the complete block of I_{Ca} by nicardipine in the experimental solution (see Drugs and solutions) because I_{Ca} is increased by hyposmotic stretch, as shown in our previous report [49]. At potentials more positive than +40 mV, the current exhibited time-dependent decay (Fig. 1B). The *I/V* relation of instantaneous current showed an outwardly rectifying shape which was not changed when the external sodium chloride was replaced by NMDG chloride (Fig. 1C). The reversal potential with external NaCl solution was 10.5 ± 0.5 mV (n = 5) and it was not significantly different from that obtained with NMDG chloride solution (10.0 ± 0.9 mV, P>0.05).

When $[Cl^-]_o$ was reduced from 86 mM to 46, 26 and 16 mM by replacement with sodium gluconate, the amplitude of the outward current was decreased while the inward current was not significantly changed (Fig. 2A). The reversal potential (E_{rev}) was shifted in the positive direction by decreasing $[Cl^-]_o$, i.e. 10.7 ± 1.2, 23.3 ± 2.5, 32.4 ± 2.8 and 38.7 ± 2.6 mV at 86, 46, 26 and 16 mM $[Cl^-]_o$, respectively (filled circles in the inset of Fig. 2B, n = 6). This result suggests that the hyposmotic-swelling-activated current was induced by the activation of an anion channel permeable to Cl^-. However, at each value

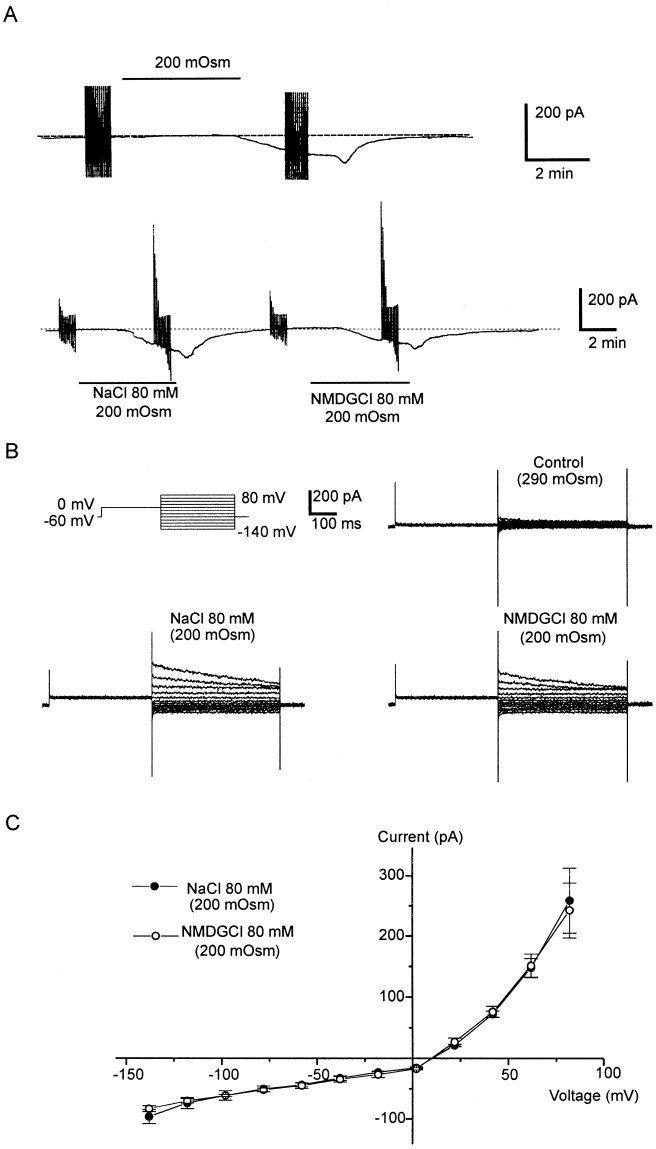

Fig. 1A–C Activation of anionic current in single gastric myocytes of guinea-pig by hyposmotic swelling. **A** At –60 mV, inward current was activated by applying hyposmotic bath solution (200 mosmol/l) when the bath solution contained either NaCl or *N*-methyl-D-glucamine chloride (*NMDGCl*). **B** Basal currents elicited by step pulses with the cell bathed in isosmotic solution 5 min after breakthrough into the whole-cell configuration and anionic currents elicited by osmotic swelling that was induced by a hyposmotic challenge in the same cell. **C** The *I/V* relation of difference currents obtained by digital subtraction of basal currents from hyposmotic-swelling-induced currents. *Symbols* represent mean ± SE (n = 5)

of $[Cl^-]$, E_{rev} did not exactly correspond to the E_{Cl} calculated from the Nernst equation (E_{Cl} = RT/F In $[Cl]_i$ /$[Cl]_o$, i.e. 11.5, 27.3, 41.6 and 53.9 mV for respective concentrations, line in the inset of Fig. 2).

There is the possibility that replacement of extracellular Cl^- results in a decrease in the intracellular Cl^- activity, especially after activation of I_{Cl} that is inward at the holding potential. This might also shift the actual Nernst po-

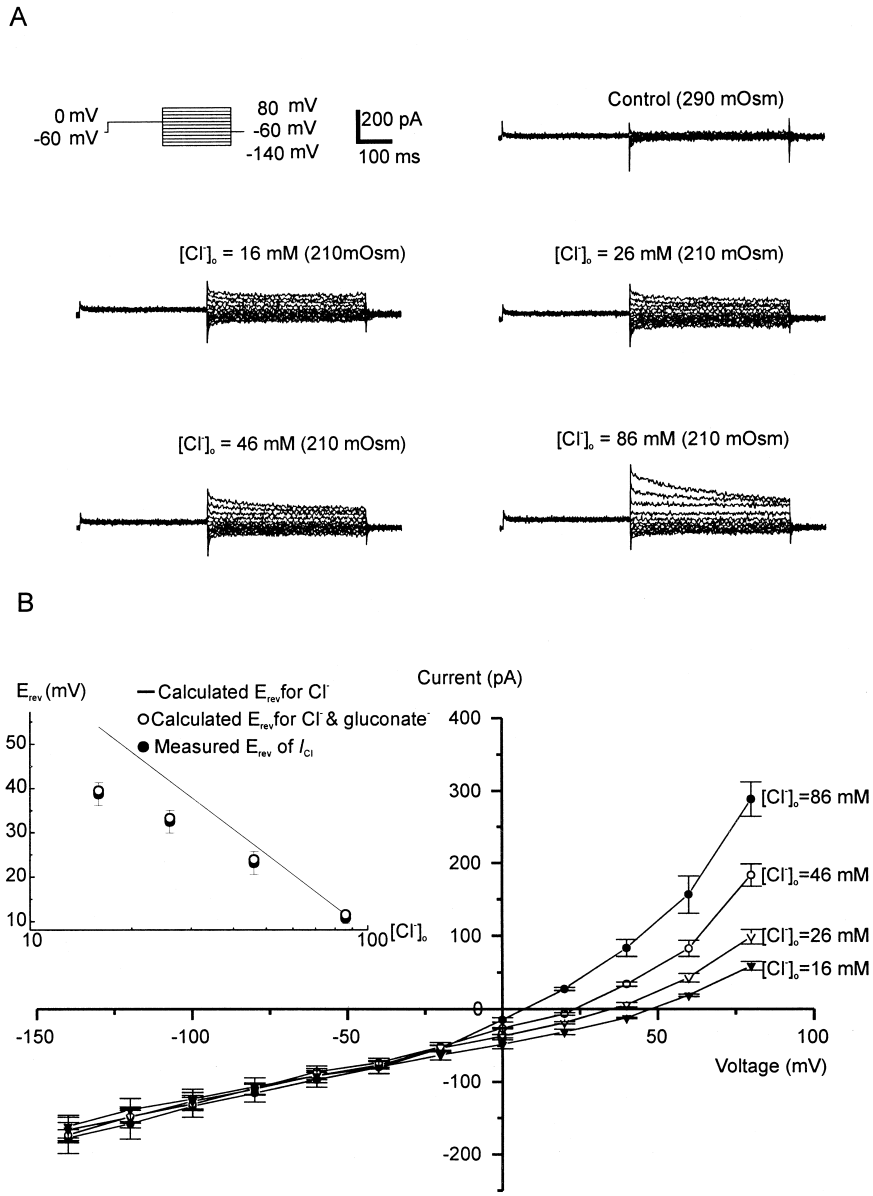

Fig. 2A, B Effects of $[Cl^-]_o$ on the *I/V* relation of hyposmotic-swelling-activated current. **A** Basal currents and hyposmotic-swelling-activated currents elicited by step pulses at various $[Cl^-]_o$. **B** The *I/V* relations of hyposmotic-swelling-activated current at various $[Cl^-]_o$ (n = 6). *Inset* shows the relation between measured reversal potentials and reversal potentials calculated according to the Nernst equation.

tentials to values that are more hyperpolarized than would be calculated from the known values of $[Cl^-]$. However, the difference is also likely to be due to a significant permeability to gluconate, which is used as the substitute for Cl^- in this experiment. As can be seen in Fig. 3, gluconate can pass through the anion channel and the relative permeability was calculated to be 0.18 compared with that of Cl^-. Considering the permeability of gluconate, the expected E_{rev} could be recalculated using Goldman-Hodgkin-Katz equation {GHK equation, $E_{Cl} = RT/F$ In $(P_{Cl}[Cl]_i + P_{gluconate}[gluconate]_i)/(P_{Cl}[Cl]_o + P_{gluconate}[gluconate]_o)$} and the results are 11.5, 23.7 33.0 and 39.5 mV, respectively (open circles in the inset of Fig. 2). These values correspond well with the measured values of E_{rev} for the osmotic-swelling-induced current in this experiment. The above results suggest that this outwardly rectifying current in gastric myocytes is mostly due to the chloride current activated by hyposmotic cell swelling (i.e. I_{Cl}).

Ionic selectivity

The anion selectivity of the channel was examined with the cells in hyposmotic bathing solutions in which Cl^- ions were replaced with other anionic species. Instantaneous *I/V* relations were obtained with a voltage-ramp protocol between +80 and –140 mV (–0.44 V/s). Figure 3 shows a representative *I/V* relation of volume-sensitive anion currents when extracellular Cl^- was subsequently replaced with the same concentration of I^-, Br^-, F^- or $gluconate^-$. From the *I/V* curves obtained at maximal current activation, the E_{rev} values were evaluated. Ion permeabilities relative to those of Cl^- ions were calculated from E_{rev} values using the GHK equation under biionic conditions ($E_{Cl} = RT/F$ In $P_{Cl}[Cl]_i/P_X[X]_o$), assuming that the currents were carried solely by anions and that the $[Cl^-]_i$ would not change during the superfusion of hyposmotic solution (Table 1). The sequence of relative

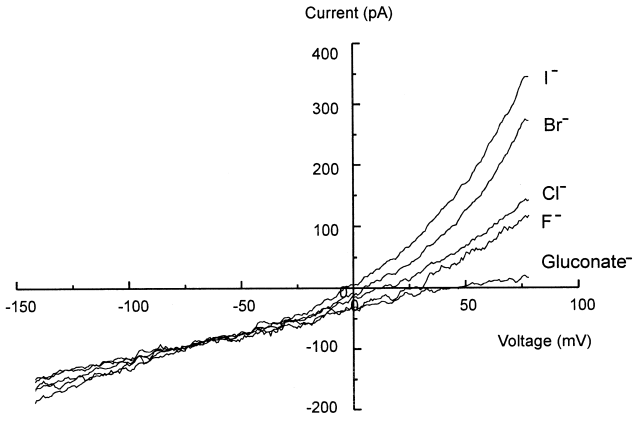

Fig. 3 Effects of anion substitution on *I/V* relations of volume-sensitive currents measured by a voltage ramp stimulus. A voltage ramp from +80 to –140 mV was applied after steady current activation was observed in osmotically swollen single gastric myocytes. Cells were superfused with hyposmotic medium in which NaCl was replaced with the sodium salt of the anions indicated.

Table 1 Effects of Cl^- ion replacement upon reversal potentials (E_{rev}) of volume-sensitive anion currents in gastric myocytes of guinea-pig. E_{rev} was obtained from *I/V* plots. Relative ion permeabilities (P_x/P_{Cl}) were calculated from the Goldman-Hodgkin-Katz equation as follows, where *x* is the replacement ion: $E_{rev}=RT/F$ In $\{[Cl]_i+(P_x/P_{Cl})\ [x]_i\}/\{[Cl]_o+(P_x/P_{Cl})[x]_o\}$, and where *R*, *T* and *F* have their usual thermodynamic meanings

Anion	E_{rev} (mV)	P_x/P_{Cl}	*n*
I^-	–3.3±0.9	1.80±0.06	6
Br^-	4.84±0.8	1.31±0.04	6
F^-	15.8±1.7	0.85±0.05	6
$Gluconate^-$	55.0±1.3	0.18±0.01	6
Cl^-	8.4±0.9	1	6

permeability obtained was I^- (1.80 ± 0.06) > Br^- (1.31 ± 0.04) > Cl^- (1.0) > F^- (0.85 ± 0.05) > gluconate$^-$ (0.18 ± 0.01). However, there still remains the uncertainty of $[Cl^-]_i$ and neither could we rule out the possibility that the substituting anions accumulate in the cytoplasm during the superfusion.

Sensitivity to Cl^- channel blockers

The effects of DIDS and niflumic acid, known to be Cl^- channel blockers [6, 7, 25, 33, 48], on I_{Cl} were tested. DIDS (100 μM) markedly suppressed the current (Fig. 4A) in a voltage-dependent manner; the outward current was significantly more susceptible to DIDS compared with the inward current ($P<0.01$, Fig. 4B, C). Outward current at +80 mV was suppressed by 92.3 ± 1.8% but inward current at –80 mV was suppressed only by 41.6 ± 13.0% in the presence of DIDS (Fig. 4C). In the presence of DIDS, the decay of I_{Cl} under highly depolarized conditions was not observable. Niflumic acid (10 μM) also inhibited the current (Fig. 4A) and equally affected both outward and inward currents (Fig. 4B). Outward current at +80 mV was suppressed by 60.8 ± 8.2% and inward current at –80 mV was suppressed by 65.3 ± 8.0% in the presence of niflumic acid (Fig. 4C).

Direct inhibition of I_{Cl} by arachidonic acid

A direct inhibitory effect of arachidonic acid on outwardly rectifying volume-sensitive I_{Cl} in human epithelial cells, human endothelial cell, the HSG cloned cell line, rat hepatocytes, airway epithelia and osteoblast-like cells has been reported [4, 11, 13, 21, 27, 35, 44]. We have also found that 25 μM arachidonic acid (superfusing with the hyposmotic solution containing 25 μM arachidonic acid) significantly inhibited I_{Cl} at a holding potential of –60 mV in gastric myocytes (Fig. 5A). In the same cell, arachidonic acid inhibited I_{Cl} elicited by a ramp pulse (from +80 to –140 mV, –0.44 /s, at –60 mV of holding potential) and equally affected inward and outward current (Fig. 5 B) Arachidonic acid inhibited inward holding current (–60 mV) by 43.8 ± 5.1, 74.8 ± 3.0 and 94.0 ± 6.9% at 5, 10 and 25 μM, respectively (n = 4, Fig. 5E). The result was fitted according to the logistic function (see Data analysis) and an IC_{50} value of 5.2 ± 0.3 μM was obtained. Other unsaturated fatty acids, linoleic acid (LA) and oleic acid (OA), also inhibited I_{Cl} but stearic acid (SA), a saturated fatty acid, did not inhibit I_{Cl}. Figure 5F shows that the inhibitory potencies of unsaturated fatty acids at 30 μM were arachidonic acid (95.2 ± 2.6%) > LA (58.8 ± 2.2%) > OA (28.5 ± 2.5%).

To test the possibility that some oxygenase metabolites of arachidonic acid mediate the latter's effect, cells were pretreated with a cyclo-oxygenase inhibitor, indomethacin (10 μM), for 20 min and subsequent hyposmotic cell swelling was induced. I_{Cl} was similarly induced in the presence of indomethacin and was inhibited by 25 μM arachidonic acid with a potency similar to that of the control (93.2 ± 11.2% in control, 92.6 ± 10.3% in the presence of indomethacin at a holding potential of –60 mV; n = 5, $P>0.05$, Fig. 5C). We also tested the possible relationship between protein kinase C and the inhibitory effect of arachidonic acid [34]. Four different cells were dialysed with 1 μM chelerythrine, a protein kinase C inhibitor [17], for 15 min. In this condition, I_{Cl} was similarly induced by hyposmotic swelling and the bath application of arachidonic acid (25 μM) still inhibited I_{Cl}, as shown in Fig 5D.

Relationship between cell volume and I_{Cl}

To investigate the role of I_{Cl} in the volume regulation of smooth muscle cells, a change in cell diameter was simultaneously measured using the video image analysis system (see Materials and methods). Figure 6A shows video images of gastric myocyte under isosmotic (Aa),

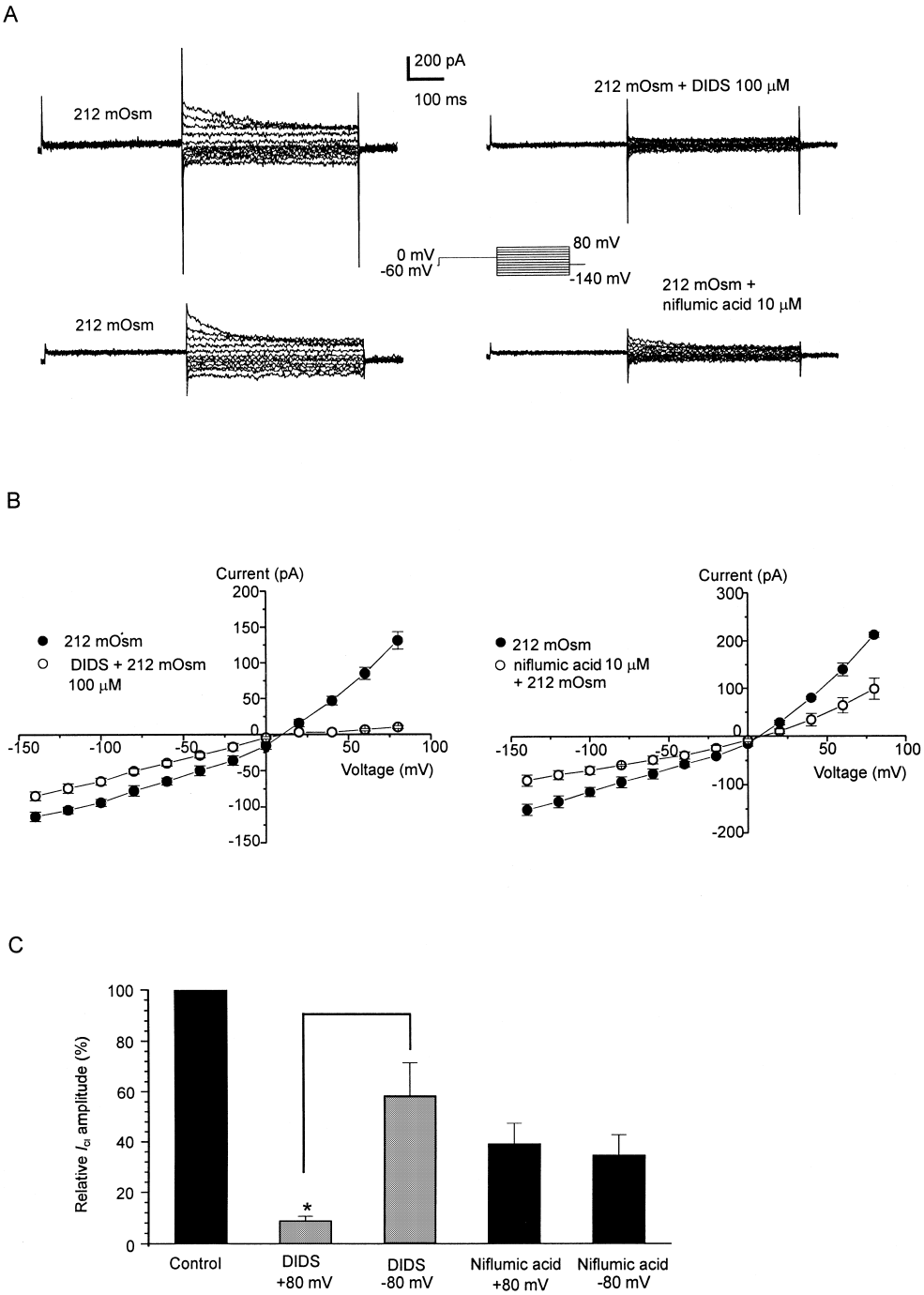

Fig. 4A–C Effects of 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (*DIDS*) and niflumic acid on I_{Cl}. **A** During steady activation of I_{Cl}, step pulses were applied in the absence and presence of DIDS or niflumic acid. **B** The *I/V* relations measured from six cells by command step pulses in the absence and presence of DIDS or niflumic acid. **C** Averaged effects of DIDS and niflumic acid on I_{Cl} at +80 and –80 mV are compared. The *asterisk* indicates a significant difference ($P<0.05$, $n = 6$)

hyposmotic (Ab) and washout (Ac) conditions. Under hyposmotic conditions, the largest diameter of the gastric myocyte increased significantly and was slowly recovered by returning to the isosmotic solution. Simultaneously recorded I_{Cl} activation is shown in Fig. 6B, where repetitive voltage ramp pulses (from +80 to –140 mV, –0.44 V/s) were applied from a holding potential of –60 mV every 20 s. In Fig. 6C, changes of cell diameter and outward peak values of I_{Cl} were measured every 20 s and plotted against time. Changes in cell diameter preceded the activation of I_{Cl} by about 1 min and, upon returning to the isosmotic condition, the decay of I_{Cl} and recovery of cell diameter occurred together. In this cell, the amplitude of I_{Cl} was suppressed by the pretreatment with niflumic acid for 3 min (from 355 pA to 143 pA) while the change of cell diameter was enhanced (from 30% to 36%) by the same treatment.

Figure 7 shows the collected results obtained from the experiment similar to that in Fig. 6. The change in cell diameter was observed 2.4 ± 0.12 min after superfusing the cells with hyposmotic solution, and was followed by I_{Cl} activation; I_{Cl} started to increase 3.4 ± 0.13 min after hyp-

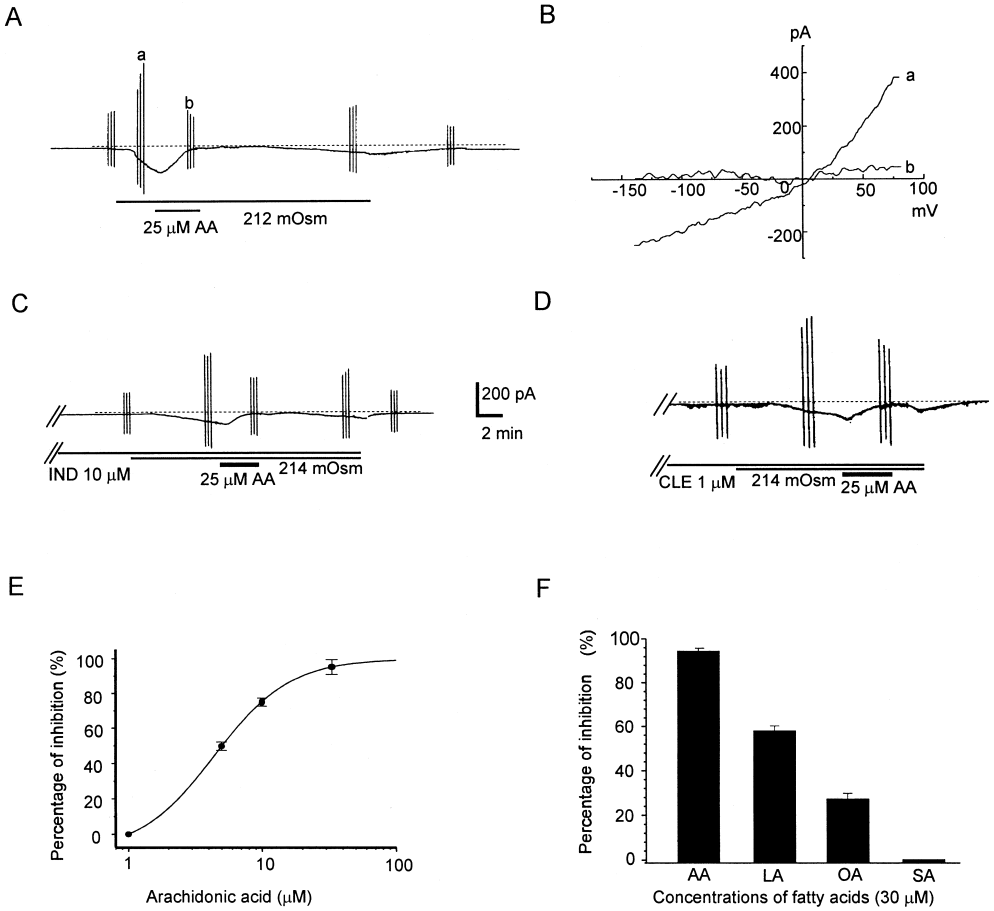

Fig. 5A–F Effect of arachidonic acid on I_{Cl}. **A** I_{Cl} was activated at –60 mV and it was completely inhibited by application of 25 μM arachidonic acid under hyposmotic conditions. **B** *I/V* curve obtained from the same cell in **A** is shown. Outward and inward currents elicited by ramp pulses (from +80 to –140 mV, –0.44 V/s) under hyposmotic conditions were inhibited by arachidonic acid. **C** Effect of arachidonic acid on I_{Cl} following pretreatment with indomethacin (*IND*) for more than 10 min. Indomethacin did not block the inhibitory effect of arachidonic acid on I_{Cl}. **D** Effect of arachidonic acid on I_{Cl} following pretreatment with chelerythrine (*CLE*) for 15 min. The inhibitory effect of arachidonic acid on I_{Cl} was not blocked by chelerythrine. **E** Dose dependence of the inhibitory effect of arachidonic acid is shown and fitted using a logistic function (see Data analysis). The half-maximally inhibitory concentration (IC_{50}) and coefficient (n) of the logistic function was 5.2 ± 0.3 μM and 1.7 ± 0.2, respectively. **F** Effects of unsaturated fatty acids and saturated fatty acid on I_{Cl}. I_{Cl} was inhibited by unsaturated fatty acids [arachidonic acid (*AA*), linoleic acid (*LA*) and oleic acid (*OA*)] but not by saturated fatty acid, stearic acid (*SA*)

osmotic superfusion. Peak outward current at +80 mV increased from 48.1 ± 8.7 pA to 227.3 ± 47.3 pA and cell volume increased by 61.7 ± 9.3% (n = 6). The recovery of ionic current and cell diameter showed similar time courses and about 4 min (4.2 ± 0.32 min) was needed for the complete recovery.

For further elucidation of the physiological role of I_{Cl}, we measured the diameter of intact gastric myocytes under isosmotic (Aa), hyposmotic (Ab) and washout (Ac) conditions (Fig. 8A). In intact gastric myocytes, relatively severe hyposmotic solution (176 mosmol/l) could elicit the significant increase in cell diameter. After returning to isosmotic conditions, we repeated the same protocol in the presence of niflumic acid (10 μM) or DIDS (100 μM).

The cell diameter was measured every 20 s and representative results are plotted against time in Fig. 8 Ba and 8 Ca. Both niflumic acid and DIDS potentiated the increase of cell diameter induced by hyposmotic stimulation (Fig. 8B, C). The maximal relative cell diameter under hyposmotic conditions was increased from 1.28 ± 0.03 to 1.38 ± 0.04 by niflumic acid (Fig. 8 Bb, n = 6, P<0.05). Pretreatment with DIDS also potentiated the increase of cell diameter from 1.25 ± 0.03 to 1.38 ± 0.03 (Fig. 8 Cb, n = 6, P<0.05). Niflumic acid or DIDS by itself had no significant effect on the cell diameter under isosmotic conditions.

Discussion

Properties of volume-sensitive Cl^- conductance

The present study suggests that gastric myocytes of the guinea-pig possess a Cl^- conductance that is activated by hyposmotic cell swelling. According to the review by Strange et al. [46], volume-sensitive anion channels found in animal cells can be categorized into three classes: (1) ClC-2, which is a member of the ClC family of

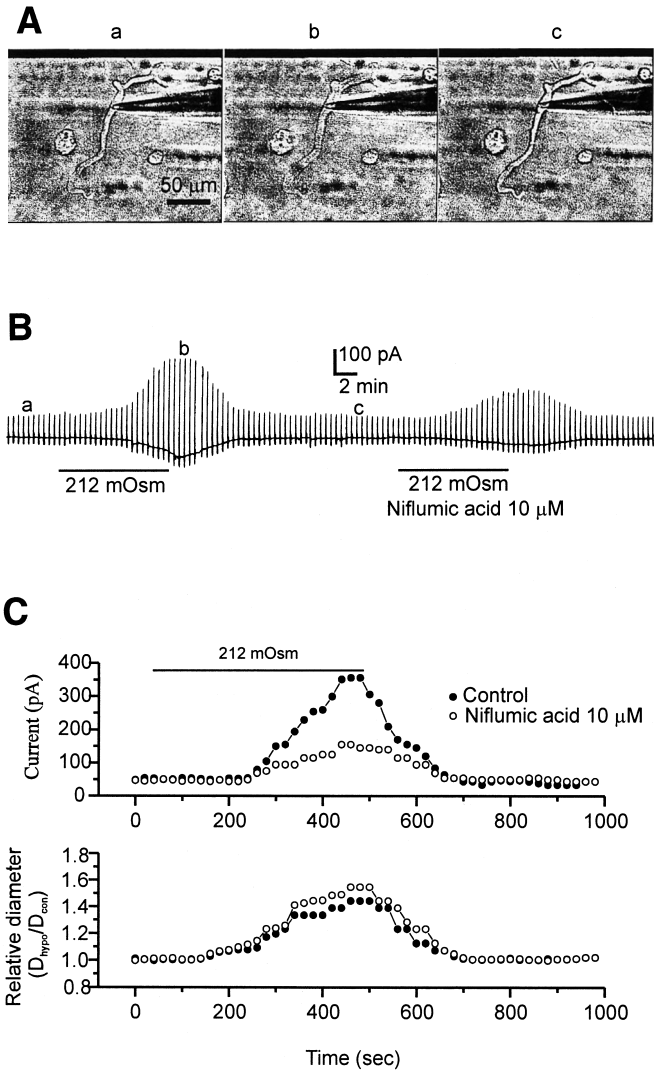

Fig. 6A–C Simultaneous measurement of cell diameter and membrane current during hyposmotic stimulation. **A** Changes of cell diameter under control conditions (**a**), during hyposmotic swelling (**b**) and washout (**c**). **B** Membrane potential was held at –60 mV and ramp pulses (from +80 to –140 mV, –0.44 V/s) were applied repetitively every 20 s. Hyposmotic stimulation slowly increased cell diameter (*a*) and was followed by the activation of inward holding current (*b*). **C** Plot of ionic current and relative cell diameter versus time (*filled circles*). Time zero implies the start of repetitive ramp pulses, about 3 min after attaining the whole-cell configuration. In the same cell, after complete recovery, hyposmotic stimulation was applied again in the presence of niflumic acid (10 mM, *open circle*)

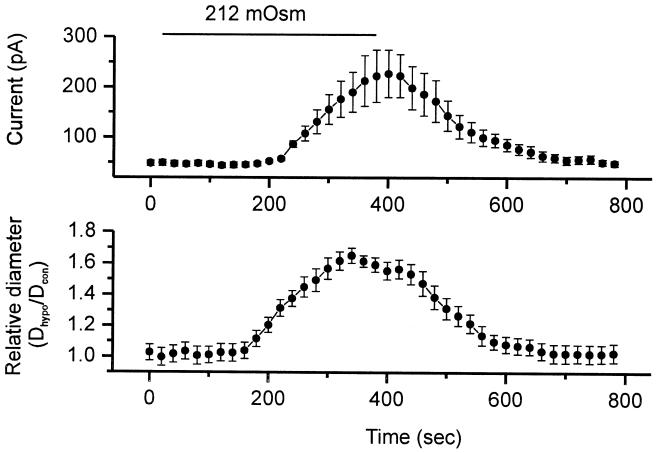

Fig. 7 I_{Cl} and changes of cell diameter by hyposmotic stimulation. In six different cells, the cell diameters and the outward peak values of I_{Cl} obtained from ramp pulses were simultaneously measured every 20 s using a protocol similar to that described in the legend to Fig. 6. Cell diameter was increased by osmotic stress and this increase was followed by I_{Cl} activation while the decay of I_{Cl} and the recovery of cell diameter occurred together

voltage-gated anion channels with very small conductance (3–5 pS); (2) an outwardly rectifying anion channel with intermediate conductance (40–50 pS); and (3) a large-conductance Cl^- channel or "maxi" channel (200–400 pS) with a linear *I/V* relation. In addition to being activated by cell swelling, the "maxi" channel is also activated by patch excision, protein kinase C activation, and cytochalasin derivatives [45].

In smooth muscle, volume-sensitive anion channels have only been reported to occur in cultured vascular smooth muscle cell lines [20] and the properties of Cl^- channels activated by osmotic swelling were similar to those reported here. The volume-sensitive anion channels observed in this experiment could be classified as volume-sensitive outwardly rectifying anion channel (VSOAC) because the outwardly rectifying *I/V* relation, the rank of relative permeability of various anions and the prominent inhibitory effects of unsaturated fatty acids coincide with the general characteristics of VSOAC in other cells [46]. The anion permeability sequence determined from the values of E_{rev} was $I^- > Br^- > Cl^- >$ gluconate$^-$ (Table 1, Fig. 3) and this sequence is in good agreement with the permeability sequence of volume-sensitive anion channels reported in connection with a number of other kinds of cells such as rat osteoblast-like cells, human epithelial cells, human promyelocytic leukemic cells (HL-60) and rabbit cardiac myocytes [12, 15, 27].

I_{Cl} observed in guinea-pig gastric myocytes seems to be independent of intracellular calcium and the calcium-dependent chloride current reported to occur in other smooth muscle cells [1, 10, 19, 26, 28, 32, 37] was not considered here since the pipette solution contained 10 mM EGTA, and 5 μM nicardipine was added to the bath solution.

I_{Cl} observed in gastric myocytes was sensitive to two structurally unrelated Cl^- channel blockers (Fig. 4). Blockade produced by the stilbene derivative DIDS was notably voltage dependent, the outward current being more prominently suppressed than inward currents. Unlike DIDS, both inward and outward currents were equally susceptible to blockade by niflumic acid and the time-dependent decay was still observed at high levels of depolarization (Fig. 4A). The effects of DIDS and niflumic acid observed in this study of gastric myocytes were very similar to the pharmacological properties of volume-sen-

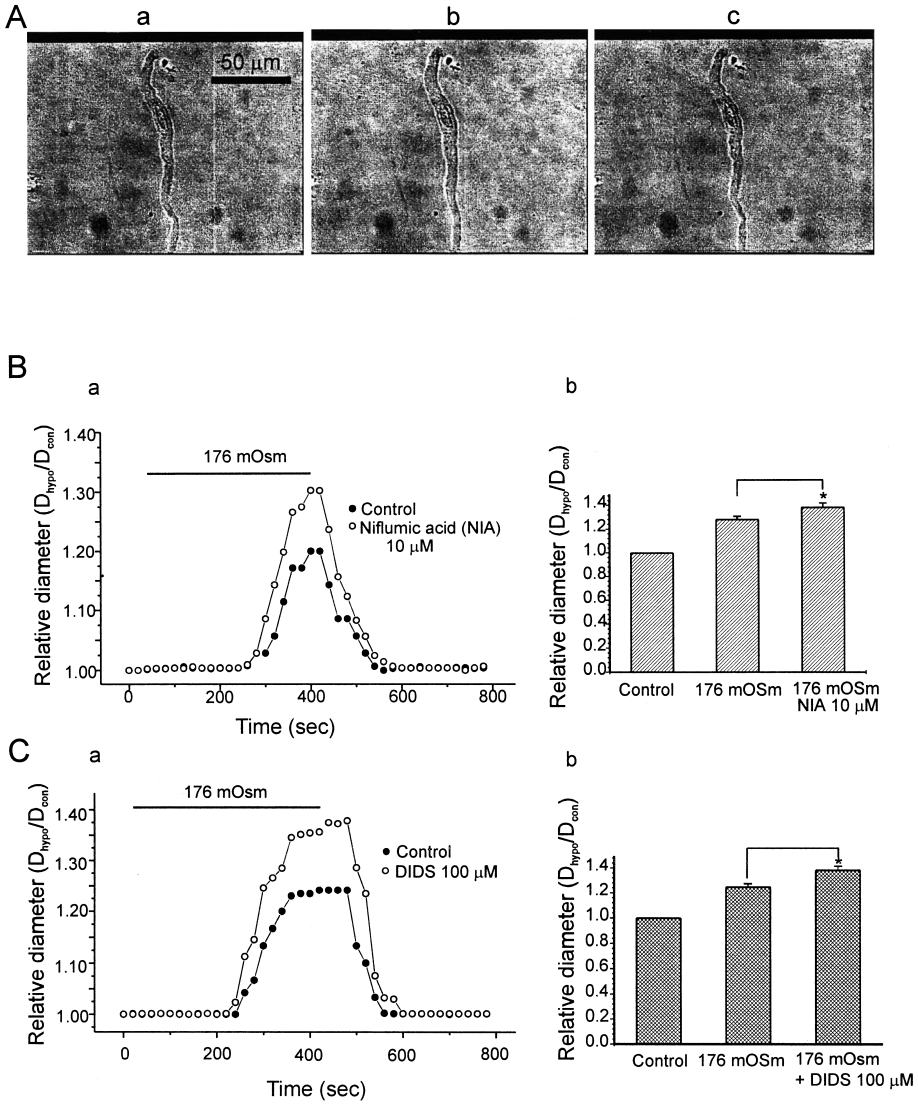

Fig. 8A–C Effects of Cl^- channel blockers, DIDS and niflumic acid, on the hyposmotic-solution-induced increase of the volume of intact, single gastric myocytes. **A** Images of single gastric myocyte under control conditions (290 mosmol/l, **a**), in the presence of hyposmotic solution (176 mosmol/l, **b**) and washout (**c**). Cell diameter was measured every 20 s using video image analysis and is plotted as the change of relative diameter as a function of perfusion time. **B** Effect of niflumic acid on the hyposmotic-solution-induced increase of cell diameter. **C** Effect of DIDS on the hyposmotic-solution-induced increase of cell diameter. Collective results from six different cells are plotted as bar graphs (*Bb* and *Cb*, mean ± SEM, * $P<0.05$)

sitive chloride currents reported to occur in cultured epithelial cells or rat osteoblast-like cells [12, 27]. In these reports, outward currents were more prominently blocked compared to the inward current by the stilbene derivatives (i.e. DIDS, SITS), while the diphenylcarboxylate (DPC) derivatives (i.e. DPC, NPPB) inhibited both inward and outward currents. Gosling et al. [12] explained that the negatively charged blocker (stilbene derivatives) would be more easily driven into the blocking site when the membrane potential is more depolarized. However, same explanation cannot be applied to the effect of niflumic acid, which is also negatively charged. Niflumic acid may act as a modulator of the channel gating process rather than as a direct blocker, similar to NPPB [2].

Effects of arachidonic acid on I_{Cl}

Unsaturated fatty acids, including arachidonic acid, are a major component of cell membrane phospholipid [23]. Various modulatory effects of unsaturated fatty acids on ion channels have been reported [13, 21, 34, 39]. In our study, prominent inhibitory effects of arachidonic acid on I_{Cl} were also observed (Fig. 5) and the half-maximally effective concentration (IC_{50}, 5.2 ± 0.3 μM) measured is slightly lower than those reported in studies of other cell preparations, i.e. 10.4 μM for rat osteoblast-like cells [13] and 8 μM for human epithelial cells [27].

There is the possibility that some indirect signalling pathways mediate the effect of arachidonic acid on I_{Cl}. In this study, the effects of indomethacin and chelerythrin have been examined to exclude the cyclo-oxygenase pathway and protein kinase C pathway [36]. Effects of other enzymatic inhibitors of lipoxygenase or the cytochrome P-450 pathway were not tested. However, it is conceivable that the effect of arachidonic acid is a direct one, as the other unsaturated fatty acids (LA, OA) were also significantly inhibitory (Fig. 5F). Previous studies also support our results: Kubo and Okada [27] and Gosling et al. [13] also concluded that osmotic-swelling-activated, outwardly rectifying Cl^- current was directly inhibited by arachidonic acid.

This result suggests that arachidonic acid and other unsaturated fatty acids may affect ion channel activity by changing the biophysical properties of the lipid membrane of gastric myocytes. It is known that the activities of various membrane proteins including ion channels are affected by the properties of the membrane lipid, i.e. the electrical charge of the hydrophilic group, the length of the acyl chain, the degree of saturation and membrane fluidity [5, 43]. The length of the acyl chain can affect ion channel activity by changing the thickness of the membrane [9, 22].

Physiological relevance

The ubiquitous ability to regulate volume is of fundamental importance to living cells. In epithelial cells, which normally experience large changes in osmolarity, volume-dependent ion transport systems play a key role in transcellular solute transport [30]. In non-epithelial cells, such as the gastric myocyte, these volume-dependent ion transport systems are probably involved in the cell volume regulation that occurs both during growth and differentiation, and also following the action of external stimuli which generate net fluxes of electrolytes and water [18, 40].

Cell volume regulation is known to involve the functioning of a variety of ion channels and transporters. Many studies have recognized that cell volume regulation under hyposmotic conditions may be accomplished by the separate activation of K^+ and Cl^- conductances, which allow effluxes of KCl and osmotically obliged water in a variety of cell species [14, 38, 47]. However, in this experiment, we did not investigate the effect of hyposmotic cell swelling on K^+ conductance. In our experiment, cell volume was increased in hypotonic solution and this increase was potentiated by Cl^- channel blockers in intact myocytes (Fig. 8). As shown in Figs. 6 and 7, a cell volume increase was followed by Cl^- current activation under whole-cell patch-clamp conditions. These results suggest that hyposmotic-cell-swelling-activated Cl^- current may contribute to cell volume regulation as one mechanisms in gastric myocytes. In fact, in intact gastric myocytes, it was more difficult to elicit a cell swelling by the usual hyposmotic stress used in whole-cell clamp conditions and the relatively severe hyposmotic solution (176 mosmol/l) induced an increase in cell diameter of only about 25% (Fig. 8). Such a difference suggests the presence of well-regulated ion efflux mechanisms as the "isovolumetric regulation process" in intact gastric myocytes, and under whole-cell clamp conditions the mechanisms might not be managed the constantly loaded concentration gradient between the pipette solution and bath solution.

Although the involvement of swelling-induced Cl^- channel activity in volume regulation is well recognized, its potential roles in other physiological functions are yet to be determined. In cultured kidney epithelial cells, the Cl^- conductance was increased by mechanical stress as well as by osmotic swelling [40]. The gastrointestinal organ will also undergo frequent stimulations by mechanical stretch. Although it has not been tested whether the I_{Cl} can be activated during membrane stretch without osmotically induced swelling of smooth muscle cells, such an investigation might provide another physiological role of I_{Cl}, a sensing mechanism for mechanical stretch.

Acknowledgement This study was supported by grant no. 961-0701-010-1 from the Korean Science and Engineering Foundation and grant no. SBRI C-95-002-2 from the Samsung Foundation.

References

1. Akbarali HI, Giles WR (1993) Ca^{2+} and Ca^{2+}-activated Cl^- currents in rabbit oesophageal smooth muscle. J Physiol (Lond) 460:117–133
2. Alton EWFW, Williams AJ (1992) Modification of gating of an airway epithelial chloride channel by 5-nitro-2 (3-phenylpropylamino) benzoic acid (NPPB). J Membr Biol 128: 141–151
3. Amédée T, Benham CD, Bolton TB, Byrne NG, Large WA (1990) Potassium, chloride and non-selective cation conductance opened by noradrenaline in rabbit ear artery cells. J Physiol (Lond) 423:551–568
4. Anderson MP, Welsh MJ (1990) Fatty acids inhibit apical membrane chloride channels in airway epithelia. Proc Natl Acad Sci USA 87:7334–7338
5. Anthony C, Donald LM (1986) How bilayer lipids affect membrane protein activity. Trends Biochem Sci 11:331–335
6. Bakhramov A, Fenech C, Bolton TB (1995) Chloride current activated by hypotonicity in cultured human astrocytoma cells. Exp Physiol 80:373–389
7. Boese SH, Kinne RKH, Wehner F (1996) Single-channel properties of swelling-activated anion conductance in rat inner medullary collecting duct cells. Am J Physiol 271: F1224–F1233
8. Chan HC, Fu WO, Chung YW, Huang SJ, Chan PSF, Wong PYD (1994) Swelling-induced anion and cation conductances in human epididymal cells. J Physiol (Lond) 478:449–460
9. Cornea RL, Thomas DD (1994) Effects of membrane thickness on the molecular dynamics and enzymatic activity of reconstituted Ca^{2+}-ATPase. Biochemistry 33:2912–2920
10. Droogmans G, Callewaert G, Declerck I, Casteels R (1991) ATP-induced Ca^{2+} release and Cl^- current in cultured smooth muscle cells from pig aorta. J Physiol (Lond) 440:623–634
11. Fatherazi S, Izutsu KT, Wellner RB, Belton CM (1994) Hypotonically activated chloride current in HSG cells. J Membr Biol 142:181–193
12. Gosling M, Smith JW, Poyner DR (1995) Characterization of a volume-sensitive chloride current in rat osteoblast-like (Ros 17/2.8) cells. J Physiol (Lond) 485:671–682
13. Gosling M, Poyner DR, Smith JW (1996) Effects of arachidonic acid upon the volume-sensitive chloride current in rat osteoblast-like (ROS 17/2.8) cells. J Physiol (Lond) 493: 613–623
14. Grinstein S, Foskett JK (1990) Ionic mechanisms of cell volume regulation in leukocytes. Annu Rev Physiol 52:399–414
15. Hagiwara N, Masuda H, Shoca M, Irisawa H (1992) Stretch-activated anion currents of rabbit cardiac myocytes. J Physiol (Lond) 456:285–302
16. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391:85–100
17. Herbert JM, Augereau JM, Gleye J, Maffrand JP (1990) Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun 172:993–999

18. Hoffman EK, Simonsen LO (1989) Membrane mechanisms in volume and pH regulation in vertebrate cells. Physiol Rev 69: 315–382
19. Hogg RC, Wang Q, Large WA (1993) Time course of spontaneous calcium-activated chloride currents in smooth muscle cells from the rabbit portal vein. J Physiol (Lond) 464:15–31
20. Holevinsky KO, Fan Z, Frame M, Makielski JC, Groppi V, Nelson DJ (1994) ATP-sensitive K^+ channel opener acts as a potent Cl^- channel inhibitor in vascular smooth muscle cells. J Membr Biol 137:59–70
21. Hwang TC, Guggino SE, Guggino WB (1990) Direct modulation of secretory chloride channels by arachidonic and other cis unsaturated fatty acids. Proc Natl Acad Sci USA 87: 5706–5709
22. In't Veld G, Driessen AJM, Op den Kamp JAF, Konongs WN (1991) Hydrophobic membrane thickness and lipid-protein interactions of the leucine transport system of *Lactococcus lactis*. Biochim Biophys Acta 1065:203–212
23. Irvine RF (1982) How is the level of free arachidonic acid controlled in mammalian cells? Biochem J 204:3–16
24. Isenberg G, Klöckner V (1982) Calcium tolerant ventricular myocytes prepared by pre-incubation in a "K-B medium". Pflügers Arch 424:431–438
25. Kelly MEM, Dixon SJ, Sims SM (1994) Outwardly rectifying chloride current in rabbit osteoclasts is activated by hyposmotic stimulation. J Physiol (Lond) 475:377–389
26. Klöckner U, Isenberg G (1991) Endothelin depolarizes myocytes from porcine coronary and human mesenteric arteries through a Ca-activated chloride current. Pflügers Arch 418:168–175
27. Kubo M, Okada Y (1992) Volume-regulatory Cl^- channel currents in cultured human epithelial cells. J Physiol (Lond) 456: 351–371
28. Lamb FS, Volk KA, Shibata EF (1994) Calcium-activated chloride current in rabbit coronary artery myocytes. Circ Res 75:742–750
29. Lang F, Busch GL, V∧lkl H, H<4,75>ussinger D (1995) Cell volume: a second messager in regulation of cellular function. News Physiol Sci 10:18–22
30. Larson N, Spring KR (1987) Volume regulation in epithelia. Curr Topics Membr Trans 30:105–123
31. Lewis SA, Donaldson P (1990) Ion channel and cell volume regulation: chaos in an organized system. News Physiol Sci 5: 112–119
32. Loirand G, Pacaud P, Baron A, Mironneau C, Mironneau J (1991) Large conductance calcium-activated non-selective cation channel in smooth muscle cells isolated from rat portal vein. J Physiol (Lond) 437:461–475
33. Meyer K, Korbmacher C (1996) Cell swelling activates ATP-dependent voltage-gated chloride channels in M-1 mouse cortical collecting duct cells. J Gen Physiol 108:177–193
34. Nagano N, Imaizumi Y, Watanabe M (1995) Modulation of calcium channel currents by arachidonic acid in single smooth muscle cells from vas deferens of the guinea-pig. Br J Pharmacol 116:1887–1893
35. Nilius B, Sehrer J, Droogmans G (1994) Permeation properties and modulation of volume-activated Cl^- currents in human endothelial cells. Br J Pharmcol 112:1049–1056
36. Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334: 661–665
37. Ohta T, Ito S, Nakazato Y (1993) Chloride currents activated by caffeine in rat intestinal smooth muscle cells. J Physiol (Lond) 465:149–162
38. Okada Y, Hazama A (1989) Volume regulatory ion channels in epithelial cells. News Physiol Sci 4:238–242
39. Ordway RW, Petrou S, Kirber MT, Walsh JV Jr, Singer JJ (1995) Stretch activation of a toad smooth muscle K^+ channel may be mediated by fatty acids. J Physiol (Lond) 484:331–337
40. Orlov SN, Resink TJ, Bernharadt J, Buhler FR (1992) Volume-dependent regulation of sodium and potassium fluxes in cultured vascular smooth muscle cells: dependence on medium osmolality and regulation. J Membr Biol 129:199–210
41. Pacaud P, Loirand G, Mironneau C, Mironneau J (1989) Noradrenaline activates a calcium-activated chloride conductance and increases the voltage-dependent calcium current in cultured single cells of rat portal vein. Br J Pharmacol 97: 139–146
42. Pacaud P, Loirand G, Baron A, Mironneau C, Mironneau J (1991) Ca^{2+} channel activation and membrane depolarization mediated by Cl^- channels in response to noradrenaline in vascular myocytes. Br J Pharmacol 104:1000–1006
43. Robert ET Jr, Anthony C, Donald L M (1986) Reconstituted human erythrocyte sugar transporter activity is determined by bilayer lipid head groups. Biochemistry 25:3709–3718
44. Sakai H, Kakinohi B, Diener M, Takeguchi N (1996) Endogenous arachidonic acid inhibits hypotonically-activated Cl^- channels in isolated rat hepatocytes. Jpn J Physiol 64:311–318
45. Schwiebert EM, Mills JW, Stanton BA (1994) Actin-based cytoskeleton regulates a chloride channel and cell volume in a renal cortical collecting duct cell line. J Biol Chem 269:7081–7089
46. Strange K, Emma F, Jackson PS (1996) Celluar and molecular physiology of volume-sensitive anion channels. Am J Physiol 270:C711–C730
47. Ulb J, Murer H, Kolb HA (1988) Hypotonic shock evokes opening Ca^{2+}-activated K channels in opossum kidney cells. Pflügers Arch 412:551–553
48. Voets T, Wei L, De Smet P, Van Driessche W, Eggermont J, Droogmans G, Nilius B (1997) Downregulation of volume-activated Cl^- currents during muscle differentiation. Am J Physiol 272:C667–C674
49. Xu WX, Kim SJ, Kim SJ, So I, Kang TM, Rhee JC, Kim KW (1996) Effect of stretch on calcium channel currents recorded from the antral circular myocytes of guinea-pig stomach. Pflügers Arch 432:159–164