VOLUME-REGULATORY $\text{Cl}^-$ CHANNEL CURRENTS IN CULTURED HUMAN EPITHELIAL CELLS

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SUMMARY

1. During osmotic swelling, cultured human small intestinal epithelial cells (Intestine 407) exhibited activation of large $\text{Cl}^-$ currents under the patch-clamp whole-cell configuration. The volume-sensitive $\text{Cl}^-$ conductance was independent of intracellular Ca$^{2+}$ and cyclic AMP.

2. The anion permeability sequence of the current was $\text{SCN}^- > \text{I}^- > \text{Br}^- > \text{Cl}^- > \text{F}^- > \text{gluconate}^-$, corresponding to Eisenman's sequence I.

3. $\text{Cl}^-$ currents were instantaneously activated by command pulses in a range of $-120$ to $+45$ mV. At potentials more positive than $+50$ mV the current showed a time-dependent inactivation. This inactivation was accelerated by increased depolarization. The instantaneous current--voltage relationship rectified in the outward direction.

4. A stilbene-derivative $\text{Cl}^-$ channel blocker, 4-acetamido-4'-isothiocyanostilbene (SITS), inhibited the $\text{Cl}^-$ current at micromolar concentrations. SITS facilitated inactivation at positive potentials. Outward currents were more prominently suppressed by SITS than inward currents. The concentrations required for 50% inhibition ($IC_{50}$) of outward and inward currents were 1.5 and 6 $\mu\text{M}$, respectively. The outward and inward currents were equally inhibited by a carboxylate analogue $\text{Cl}^-$-channel blocker, 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) or diphenylamine-2-carboxylate (DPC) at higher doses ($IC_{50} = 25$ for NPPB or 350 $\mu\text{M}$ for DPC). Inactivation kinetics at large depolarizations was not affected by NPPB or DPC.

5. The $\text{Cl}^-$ current was blocked by an unsaturated fatty acid, arachidonic acid ($IC_{50} = 8 \mu\text{M}$). Arachidonic acid was still effective in the presence of inhibitors of lipoxygenase (nordihydroguaiaretic acid, 10 $\mu\text{M}$), cyclo-oxygenase (indomethacin, 10 $\mu\text{M}$) and protein kinase C (polymyxin B, 30 $\mu\text{M}$). The $\text{Cl}^-$ current was also sensitive to another cis unsaturated fatty acid, oleic acid, which is not a substrate for oxygenases. A trans isomer of oleate, elaidic acid, and a saturated fatty acid, palmitic acid, were ineffective.

6. Single Intestine 407 cells exposed to a hypotonic solution showed a regulatory volume decrease after initial osmotic swelling. The volume regulation was abolished by SITS, NPPB, arachidonate and oleate, but not by elaidate and palmitate.

7. It is concluded that outwardly rectifying $\text{Cl}^-$ channels, which are sensitive to
arachidonic acid, are activated upon osmotic swelling and involved in the subsequent cell volume regulation.

INTRODUCTION

Cell volume regulation under hypotonic conditions may be accomplished by separate activation of conductive K+ and Cl− pathways, or by other, usually electroneutral, K+ efflux mechanisms, which allow effluxes of KCl and osmotically obliged water, in a variety of cell species (see Hoffman & Simonsen, 1989; Okada & Hazama, 1989; Grinstein & Foskett, 1990 for recent reviews). In a human intestinal epithelial cell line (Intestine 407), parallel activation of the K+ and Cl− conductances has been directly observed by both two-microelectrode voltage-clamp and whole-cell patch-clamp studies (Hazama & Okada, 1988). An increase in the cytosolic free Ca2+ concentration is responsible for activation of the volume-regulatory K+ channel current in the epithelial cell (Hazama & Okada, 1990a). The properties of volume-sensitive Cl− currents have been characterized to some extent (Cahalan & Lewis, 1988; Hazama & Okada, 1988; Hudson & Schultz, 1988; McCann, Li & Welsh, 1989; Worrell, Butt, Cliff & Frizzell, 1989; Estacion, 1991; Yantorno, Carre, Cola-Prados, Krupin & Civan, 1992); however, the mechanisms by which the volume-regulatory Cl− channel is activated have not been directly determined.

In small intestinal cells, the presence of volume-sensitive Cl− conductances has been shown (Giraldez, Valverde & Sepulveda, 1988; Hazama & Okada, 1988; MacLeod & Hamilton, 1991b). Sepulveda and his collaborators have found two types outward-rectifying Cl− currents have been isolated enterocytes (Giraldez, Murray, Sepulveda & Sheppard, 1989; Sepulveda, Fargon & McNaughton, 1991). However, the volume-sensitivity of these Cl− currents is not known.

Arachidonic acid and its metabolites are known to be important mediators of physiological cellular processes (Irvine, 1982). Recently, it was shown that outwardly rectifying, intermediate-conductance Cl− channels are directly inhibited by arachidonic acid in airway epithelial cells (Anderson & Welsh, 1990; Hwang, Guggino & Guggino, 1990). Lambert (1987) provided the data of cell volume measurements which strongly suggest that the Cl− transport pathway responsible for a regulatory volume decrease is sensitive to arachidonate. It is then possible that arachidonic acid directly suppresses the volume-sensitive Cl− channel current.

In the present study, the properties of volume-sensitive Cl− currents, including their sensitivity to voltages, Cl− channel blockers and arachidonic acid, were investigated by the whole-cell patch-clamp technique in a small intestinal epithelial cell line (Intestine 407). Preliminary data were presented at The Physiological Society Meeting in Cambridge in July 1991 (Kubo & Okada, 1992).

METHODS

Cells

A human small intestinal epithelial cell line (Intestine 407), which is known to retain receptors to a variety of intestinal secretagogues (Yada & Okada, 1984; Yada, Oiki, Ueda & Okada, 1989), was cultured in Fischer medium supplemented with 10% newborn calf serum, as described previously (Hazama & Okada, 1988). Suspensions of spherical cells were prepared by detaching from the plastic substrate and culturing with agitation for 10–120 min.

The cells placed in a chamber (0.5 ml) were perfused at a flow rate of around 5 ml/min by gravity
feed from reservoirs or by hydrostatic pressure from syringes. A hypotonic challenge was made by switching the perfusate from an isotonic to hypotonic solution.

Whole-cell current recordings

The patch electrodes were fabricated from haematocrit capillaries, as described previously (Kotera, Hashimoto, Ueda & Okada, 1991). Since we had large currents, low-resistance pipettes (around 1 MΩ when filled with pipette solutions) were employed to reduce the voltage drop across the residual series resistance. The series conductance (200–600 nS) and capacitance (20–30 pF) were maximally compensated.

| TABLE 1. Composition of solutions (mM) used in whole-cell recordings |
|---------------------------------------------------------------|

A. CsCl solutions (pH 7.4–7.5)

| Pipette* | Isotonic bathing | Hypotonic bathing |
|----------|------------------|-------------------|
| CsCl     | 110              | 110               | 110               |
| MgSO₄    | 2                | 5                 | 5                 |
| Sodium gluconate | — | 3.5 | 3.5 |
| Na-HEPES | 15               | —                 | —                 |
| HEPES    | 10               | 12                | 12                |
| Tris     | —                | 8                 | 8                 |
| EGTA     | 1                | —                 | —                 |
| ATP-Na₂  | 1                | —                 | —                 |
| Mannitol | 50               | 100               | 40                |

B. KCl solutions (pH 7.3–7.4)

| Pipette* | Isotonic bathing | Hypotonic bathing |
|----------|------------------|-------------------|
| KCl      | 147              | —                 | —                 |
| Potassium aspartate | — | 9.5 | 9.5 |
| NaCl     | —                | 10                | 10                |
| Sodium gluconate | — | 84 | 84 |
| MgCl₂    | 2                | 5                 | 5                 |
| CaCl₂    | 0.03             | 1                 | 1                 |
| Na-HEPES | 5                | 6                 | 6                 |
| HEPES    | 5                | 8                 | 8                 |
| EGTA     | 0.2              | —                 | —                 |
| ATP-Na₂  | 1                | —                 | —                 |
| Mannitol | —                | 110               | 60                |

C. Choline chloride solutions (pH 7.2–7.4)

| Pipette* | Isotonic bathing | Hypotonic bathing |
|----------|------------------|-------------------|
| Choline chloride | 135 | 135 | 135 |
| MgSO₄    | 2                | 5                 | 5                 |
| HEPES    | 6                | 6                 | 6                 |
| Tris     | 6                | 4                 | 4                 |
| EGTA     | 1                | —                 | —                 |
| ATP-Na₂  | 1                | —                 | —                 |
| Mannitol | 30               | 60                | —                 |

* The pCa values were around 7.5 in the KCl pipette solution and around 9 in the CsCl and choline chloride pipette solutions, since 10 μM Ca²⁺ was found to be contaminating the distilled water in our laboratory with Ca²⁺-selective microelectrodes.

Voltage-pulses were generated by a computer-loaded pulse generator (Shoshin EM, Type OI-8). The whole-cell patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) was essentially the same as described previously (Hazama & Okada, 1988; Kotera et al. 1991). Whole-cell currents were recorded with a patch-clamp amplifier (List EPC7). The currents were converted to digital signals by a pulse-code modulator (Sony PCM-501ES) and stored on tape with a video
cassette recorder (Victor HR-085) as well as on diskettes via a personal computer (NEC PC9801DX).

The leak current component was not compensated. Junctional potentials were measured with reference to a salt bridge for different extracellular Cl\(^-\) concentrations and corrected.

Three types of solutions (CsCl, KCl and choline chloride solutions) were employed for pipette and bathing solutions (Table 1). The osmolarity of pipette solutions was set lower (by 30–40 mmol/kg H\(_2\)O) than that of isotonic bathing solutions in order to prevent spontaneous cell swelling after attaining the whole-cell mode (due to poorly diffusible cytosolic constituents: see Worrell et al. 1989). Unless otherwise stated, CsCl solutions were employed. In some experiments 0.2 mm ethylene glycol bis(\(\beta\)-aminoethyl ether)-\(N,N',N'\)-tetraacetic acid (EGTA), instead of 1 mm, was added to the CsCl pipette solution. When necessary, 5 mm 1,2-bis(2-aminophenoxy)ethane-\(N,N',N'\)-tetraacetic acid tetrapotassium salt (BAPTA) was added to the pipette solution. To reduce the extracellular Cl\(^-\) concentration, choline chloride was replaced with equiosmolar mannitol in the hypotonic chloride solution. To examine anion selectivity, the CsCl pipette solution and '\(\text{Na}^+\)' bathing solutions were employed. The Na\(^+\)X solutions were composed of (mm): 4 Tris, 6 HEPES and 135 NaSCN, NaI, NaBr, NaCl, NaF or sodium gluconate (pH 7.3).

**Single-cell size measurements**

Single-cell diameters before (\(d_a\)) and after a hypotonic challenge (\(d\)) were measured under a phase-contrast microscope (Nikon TMD) and a video monitor system; The relative cell volume is given by the third power of the diameter ratio (\(d/d_a^3\)). The cell was held by a giga-sealed patch pipette at the same location in a microscopical field during perfusion of an isotonic or hypotonic solution, which had the following composition (mm): 137.5 or 54 NaCl, 4.2 KCl, 0.9 CaCl\(_2\), 0.5 MgCl\(_2\), 20 or 26.6 mannitol, 6 Na-HEPES, and 8 HEPES (pH 7.3).

**Reagents**

4-Acetamido-4'-isothiocyanostilbene (SITS), diphenylamine-2-carboxylate (DPC), arachidonic acid (20:4, \(\text{cis-5,8,10,14-eicosatetraenoic acid}\)), oleic acid (\(\text{cis-9-octodecanoic acid}\)), elaidic acid (\(\text{cis-9-octodecanoic acid}\)), and palmitic acid (16:0, \(\text{hexadecanoic acid}\)) were obtained from Nacalai Tesque (Kyoto). Nordihydroguaiaretic acid (NDGA) and indomethacin were obtained from Sigma (St Louis, MO, USA). 5-Nitro-2-(3-phenyl-propylamino)-benzoate (NPPB) was generously provided by Drs N. Takeguchi and H. Sakai (Toyama Medical Pharmaceutical University). Ionomycin was purchased from Calbiochem (La Jolla, CA, USA). All above chemicals were added to the bathing solution from stock solutions in dimethyl sulphoxide. The vehicle alone had no effect on whole-cell currents and cell volume at the concentrations employed (less than 0.1%). Adenosine 3':5'-cyclic monophosphate (cyclic AMP, Sigma), N-(2-(methylamino)ethyl)-5'-isoquinolinesulphonamide dihydrochloride (H-8, Seikagaku Kogyo, Japan) and polymyxin B sulphate (Nacalai Tesque, Kyoto) were dissolved directly in the solutions.

All experiments were performed at room temperature (24–26 °C). The data are expressed as the means ± s.e. of the mean (\(n\), number of observations).

**RESULTS**

**Volume-sensitive chloride currents**

Under whole-cell voltage-clamp, single Intestine 407 cells equilibrated with KCl solutions (Table 1B) responded to a hypotonic challenge (86% osmolarity) with osmotic cell swelling (with no subsequent volume recovery) as well as with activation of outward currents at the equilibrium potential to Cl\(^-\) (\(E_{\text{Cl}^-} + 50\) mV) and of inward currents at the equilibrium potentials to monovalent cations (\(E_{\text{Na}^+,\text{K}^+} - 10\) mV) and K\(^+\) (\(E_{\text{K}^+} - 70\) mV), as shown in Fig. 1A. Under this condition, the outward and inward currents would represent mainly K\(^+\) and Cl\(^-\) currents, respectively (Hazama & Okada, 1988).

When cells were equilibrated with K\(^+\)-free, CsCl or choline chloride solutions (Table 1A and C), activation of large currents at both positive and negative potentials was found to take place in association with cell swelling induced by a hypotonic challenge (83% osmolarity), as shown in Fig. 1 (B and C). Since the current
Fig. 1. Current activation upon osmotic cell swelling under whole-cell recordings in single Intestine 407 cells equilibrated with the KCl (A), CsCl (B) and choline chloride solutions (C). The membrane potential was clamped alternately to three different voltages (−70, −10 and +50 mV in A, −40, 0 and +40 mV in B and C). The extracellular osmolarity was changed by superfusing with a hypotonic (at the open arrow) or isotonic solution (at the filled arrow). The zero current level is indicated by the arrowhead. The cell diameter (in μm) is given above the traces. Visible swelling always started 5–10 s prior to the onset of current activation. The data represent six (A), eleven (B) and three similar experiments (C). Under symmetrical choline chloride (C) or CsCl conditions (B), small inward currents were often observed at 0 mV (= $E_{cl}$). Small inward currents at 0 mV were also observed when the extracellular MgSO$_4$ concentration was reduced to 2 mM to attain symmetrical conditions with respect to Mg$^{2+}$, which is another candidate for the inward current carrier (duplicate experiments). Therefore, it may be inferred that the small currents at $E_{cl}$ were associated with non-equilibrium, electrokinetic phenomena during vast fluxes of water and ions across the membrane or with small deviation of the membrane potential from $E_{cl}$ presumably due to a junction (Donnan) potential between the pipette solution and the cytosol.
Fig. 2. Effects of extracellular Cl\(^-\) concentration changes (A) and anion substitutions (B) on current–voltage curves measured by ramp voltage clamp. Single Intestine 407 cells were equilibrated with the choline chloride pipette and hypotonic choline chloride bathing solutions (A) and with the CsCl pipette and hypotonic NaX bathing solutions (B). Ramp command voltages from \(-100\) to \(+100\) mV were applied for 2-4 s after the steady current activation was observed in association with steady osmotic swelling. Cl\(^-\) gradients and extracellular anion species were indicated on current traces in A and B, respectively. The ramp clamp protocol allows accurate evaluation of \(E_{\text{rev}}\) despite the history dependency of the Cl\(^-\) current amplitude (as described later). Inset, relation between the reversal potential \((E_{\text{rev}})\) and the external Cl\(^-\) concentration \([\text{Cl}^-]_o\). Each symbol represents the mean \(E_{\text{rev}}\) value of eight observations with the s.e. of the mean (vertical bar). The straight line has a 55 mV/decade slope.

TABLE 2. Reversal potentials and slope conductances of volume-sensitive anion currents under bi-ionic conditions

| Anion    | \(E_{\text{rev}}\) * (mV) | Slope conductance† (nS) | \(n\) |
|----------|-----------------------------|--------------------------|------|
| SCN\(^-\) | \(-15.4 \pm 1.0\)          | \(77.6 \pm 9.4\)         | 5    |
| I\(^-\)  | \(-11.7 \pm 0.6\)          | \(76.1 \pm 6.4\)         | 7    |
| Br\(^-\) | \(-9.2 \pm 0.6\)           | \(77.8 \pm 10.4\)        | 6    |
| Cl\(^-\) | \(-5.1 \pm 0.2\)           | \(77.1 \pm 7.4\)         | 11   |
| F\(^-\)  | \(+7.1 \pm 1.3\)           | \(62.1 \pm 10.6\)        | 7    |
| Gluconate\(^-\) | \(+51.3 \pm 3.5\) | \(34.0 \pm 7.2\) | 6    |

* Uncorrected for junction potentials (around \(+6\) mV for gluconate but \(\leq 1\) mV for other anions).
† Measured at \(E_{\text{rev}}\).

profile and the zero-current (reversal) potential were virtually independent of cationic species (Na\(^+\), Cs\(^+\) and choline\(^+\)), the current must be carried by Cl\(^-\). These observations were further complemented by measuring the reversal potentials \((E_{\text{rev}})\) at three different extracellular choline chloride concentrations with ramp command voltages (from \(-100\) to \(+100\) mV for 2-4 s) applied upon steady osmotic swelling (Fig. 2A). The \(E_{\text{rev}}\) value shifted by \(+55\) mV per a 10-fold decrease in the external Cl\(^-\) concentration (inset in Fig. 2A). This is close to the theoretical value for a Cl\(^-\)-selective channel.

Anion selectivity of the channel was examined by the ramped voltage clamp in
hypotonic bathing solutions in which Cl\(^-\) ions were totally replaced with other anionic species. From these current–voltage curves observed during maximal current activation upon steady osmotic swelling (Fig. 2B), the reversal potentials and slope conductances were evaluated (Table 2). The relative anion conductance estimated from the slope conductance was SCN\(^-\) \(\sim\) I\(^-\) \(\sim\) Br\(^-\) \(\sim\) Cl\(^-\) > F\(^-\) > gluconate\(^-\). The permeability sequence obtained from reversal potentials was SCN\(^-\) > I\(^-\) > Br\(^-\) > Cl\(^-\) > F\(^-\) > gluconate\(^-\). The corresponding normalized permeability coefficients were estimated to be 1·5, 1·3, 1·2, 1·0, 0·6 and 0·1, assuming that currents were solely carried by anions.

**Voltage dependence**

The voltage dependence of the volume-sensitive Cl\(^-\) current was first investigated by applying positive and negative pulses alternately with 20 mV increments between −120 and +120 mV (Fig. 3A) at 7 s intervals. Basal whole-cell currents in unstimulated cells under isotonic conditions were very low (<±50 pA at ±100 mV) and remained stable (Fig. 3B). After a hypotonic challenge stationary currents were instantaneously activated upon applications of voltage pulses of less than ±60 mV. The currents exhibited time-dependent inactivation at potentials more positive than +60 mV, whereas time-dependent activation of the currents appeared at potentials more negative than −60 mV (Fig. 3C). Inactivation became progressively faster as the potential was clamped to increasingly positive voltages.

Figure 4A shows tail currents measured at different voltage levels (−90 to +105 mV) after full activation of the osmotically induced current at −105 mV. The tail currents at potentials more negative than +45 mV had an instantaneous and steady profile, whereas at more positive potentials the currents rapidly inactivated after instantaneous activation (Fig. 4A). Furthermore, no time-dependent activation occurred at potentials more negative than −60 mV. In contrast, after full inactivation of the currents by large positive pulses (+100 mV), the tail currents showed time-dependent activation kinetics at negative potentials (Fig. 4B) and positive potentials less than +45 mV (Fig. 4C).

The magnitude of pulse-induced instantaneous currents was dependent upon the pre-potential level. The more negative the conditioning pulse, the greater the instantaneous current was evoked by a constant positive command potential (Fig. 4D). Such history dependency was observed even 15 s after restoring the holding potential to 0 mV (Fig. 4E). Then, the apparent time-dependent activation observed at <−60 mV with the pulse protocol alternated every 7 s (Fig. 3C and D) would have in fact represented the release from inactivation induced by previous large positive pulses. Thus, the accurate current–voltage relationship cannot be evaluated by the alternating pulse protocol (Fig. 3A) or the ramp clamp protocol (as applied in Fig. 2) because of the history dependency. Therefore, the instantaneous whole-cell current–voltage relationship was re-evaluated from the tail currents measured at different voltages after full activation by a pre-pulse of −105 mV (as in Fig. 4A). The resultant relationship showed rectification in the outward direction with slope conductances of around 120 and 40 nS at +90 and −75 mV, respectively (Fig. 5).
Fig. 3. Whole-cell Cl⁻ current responses to positive and negative pulses applied alternately to single Intestine 407 cells equilibrated with the CsCl solutions. A, the pulse protocol. B, basal currents before hypotonic stimulation in a cell dialyzed with the CsCl pipette solution (containing 1 mM EGTA). C, Cl⁻ currents upon steady swelling induced by a hypotonic challenge in the same cell as B. D, Cl⁻ currents upon steady osmotic swelling in a cell dialyzed with the CsCl pipette solution to which 5 mM BAPTA was supplemented. E, Cl⁻ currents upon peak activation induced by ionomycin (1 μM) in a cell dialyzed with the CsCl pipette solution containing 0.2 mM EGTA. F, Cl⁻ currents upon peak swelling induced by a hypotonic challenge in the cell incubated with the CsCl solution containing...
Calcium independence

Osmotic swelling induced activation of Cl\textsuperscript{−} currents irrespective of cytosolic free Ca\textsuperscript{2+} concentrations maintained with EGTA (pCa (−log [Ca\textsuperscript{2+}]) 7.5 in Fig. 1A or pCa 9 in Figs 1B and C, 2, 3C, 4 and 5). Also, essentially similar volume-sensitive Cl\textsuperscript{−} currents were observed when cytosolic Ca\textsuperscript{2+} was strongly chelated with 5 mM BAPTA (Fig. 3D). This is in good agreement with our previous results obtained by dialysing with 10 mM EGTA (Hazama & Okada, 1988).

In the presence of a Ca\textsuperscript{2+} ionophore (ionomycin, 1 μM), Cl\textsuperscript{−} currents were activated under isotonic conditions (Fig. 3E). However, the peak currents were much smaller than Cl\textsuperscript{−} currents induced by osmotic swelling, and, moreover, the kinetic pattern of Ca\textsuperscript{2+}-activated currents was quite different from that of volume-sensitive currents. Ionomycin-induced currents exhibited activation upon depolarizations and inactivation upon hyperpolarizations.

Cyclic AMP independence

When cyclic AMP (1–2 mM) was added to the choline chloride pipette solution, the basal currents under isotonic conditions were increased by more than three times (from 33±5 to 98±7 pA at +100 mV and from −23±7 to −101±13 pA at −100 mV, n = 10). In the presence of intracellular cyclic AMP neither outward nor inward currents exhibited time-dependent inactivation or activation, and the current–voltage relationship was almost linear. Upon osmotic swelling activation of large Cl\textsuperscript{−} currents was normally observed with cyclic AMP (five observations).

As shown in Fig. 3F, the magnitude and pattern of Cl\textsuperscript{−} currents activated by osmotic swelling were virtually unaffected by prior administration of an inhibitor of cyclic AMP-dependent protein kinase, H-8 (Hidaka, Inagaki, Kawamoto & Sakai, 1984).

Sensitivity to Cl\textsuperscript{−} channel blockers

Volume-sensitive Cl\textsuperscript{−} currents were rapidly suppressed by a stilbene-derivative Cl\textsuperscript{−} channel blocker, SITS, at 1–100 μM. The SITS effect was fairly reversible (about 70 % at 30 μM) after wash-out (Fig. 6A and B). In the presence of SITS, inactivation of the current was accelerated in both its rate and the threshold voltage level (Fig. 6C). The outward current was more sensitive to SITS than the inward current. The half-maximum inhibition doses (IC\textsubscript{50}) were 1.5 and 6 μM for the outward and inward currents, respectively (Fig. 7).

A carboxylate analogue Cl\textsuperscript{−} channel blocker, NPPB, also dose-dependently inhibited the current (Figs 7 and 8). The NPPB effect was fully reversible. The outward and inward currents were equally affected with an IC\textsubscript{50} of 25 μM. The inactivation kinetics was not affected by this drug. Another carboxylate analogue Cl\textsuperscript{−} channel blocker, DPC, similarly suppressed the volume-sensitive Cl\textsuperscript{−} current at high doses (IC\textsubscript{50} 350 μM) (Fig. 7).

5 μM H-8 for 20 min. The step pulses (2 s duration) up to ±120 mV (as shown in A) were applied in B–D and to ±100 mV in E and F. The zero current level is indicated by the horizontal line. The data are representative of thirty-one (B, C), five (D), and three (E, F) similar experiments.
Fig. 4. Effects of conditioning pulses on whole-cell Cl⁻ currents in single osmotically swollen Intestine 407 cells equilibrated with the CsCl solutions. Conditioning and command pulse voltages (mV) are indicated on each trace. The zero current level is indicated by the horizontal line. In D and E, the current responses of a, b and c to +100 mV command pulses were induced by the corresponding negative conditioning pulses. The data represent six (A, B), eight (C) and three experiments (D, E).

**Sensitivity to arachidonic acid**

Based on the data of cell volume measurements in Ehrlich ascites tumour cells, Lambert (1987) strongly suggested that the Cl⁻ transport pathway responsible for the regulatory volume decrease is sensitive to an unsaturated fatty acid, arachidonic
acid. We directly tested whether the volume-sensitive Cl\(^-\) channel current is inhibited by arachidonic acid in Intestine 407 cells. Arachidonic acid (25 \(\mu\)M) added in the perfusate rapidly blocked the Cl\(^-\) current (Fig. 9A). This effect was reversible. As shown in 9B, recovery was facilitated when the perfusate contained bovine serum albumin, which is known to bind fatty acids (Spector, Fletcher & Ashbrook, 1969). Arachidonic acid was effective at physiological concentrations (1–30 \(\mu\)M; Fig. 10), and the IC\(_{50}\) (8 \(\mu\)M) was comparable to the \(K_m\) (Michaelis–Menten constant) of cyclo-oxygenase (5 \(\mu\)M) and lipoxygenase (3.4–28 \(\mu\)M) (Needleman, Turk, Jakschik, Morrison & Lefkowith, 1986).

In the presence of inhibitors of cyclo-oxygenase and lipoxygenase, indomethacin and NDGA (Rainsford, 1988), arachidonic acid was still effective in inhibiting the volume-sensitive Cl\(^-\) currents (Fig. 9C). Arachidonic acid also blocked the current in cells loaded with an inhibitor of protein kinase C, polymyxin B (Mazzei, Katoh & Kuo, 1982), as shown in Fig. 9D.

Another cis unsaturated fatty acid, oleic acid, which is not a substrate for oxygenases, also blocked the volume-sensitive Cl\(^-\) current (Fig. 9E) in a dose-dependent manner (Fig. 10). In contrast, a trans isomer of oleate, elaidic acid, and a saturated fatty acid, palmitic acid, did not inhibit the Cl\(^-\) current (10–50 \(\mu\)M, three and four observations).

**Involvement in the regulatory volume decrease**

Single-cell size measurements showed that Intestine 407 cells exhibit a regulatory volume decrease after transient osmotic swelling upon a hypotonic challenge (Fig. 11A, ○), as found in the suspended cells by electronic mean cell size measurements (Hazama & Okada, 1988) and in the monolayer cells by Fura-2 concentration measurements (Okada, Hazama & Yuan, 1990).
The regulatory volume decrease was impaired by the extracellular application of a Cl⁻ channel blocker (SITS or NPPB). A cis unsaturated fatty acid, arachidonic acid or oleic acid, also inhibited the volume regulation, whereas a trans isomer of oleate, elaidic acid, had no effects (Fig. 11B). A saturated fatty acid, palmitate, also failed to block the volume regulation under hypotonic conditions (duplicate observations). Therefore, it appears that the cell volume regulation under hypotonic conditions is abolished by blocking the volume-sensitive Cl⁻ current in Intestine 407 cells.

Fig. 6. Effects of SITS on volume-sensitive Cl⁻ currents. The currents were recorded by applying alternating pulses (0 to ±40 mV) in A and B or command pulses (as in Fig. 3A) in C to single Intestine 407 cells equilibrated with the CsCl solutions. Hypotonic challenges were made at the arrow in A, and 7 to 16 min before recordings in C. The zero current level is indicated by the arrowhead (A, B) or horizontal line (C).
Fig. 7. Dose–response curves of SITS (triangles), NPPB (squares) and DPC (circles) for the inward (open symbols) and outward Cl\(^-\) currents (filled symbols). The currents were observed at \(\pm 40\) mV in single osmotically swollen Intestine 407 cells equilibrated with the CsCl solutions. The ordinate represents the percentage of peak currents at maximum inhibition against steady currents before drug applications. Each symbol represents the mean value of four to nine experiments with the s.e. of the mean (vertical bar).

Fig. 8. Effects of NPPB on volume-sensitive Cl\(^-\) currents. The currents were recorded by applying alternating pulses (0 to \(\pm 40\) mV) in A or command pulses (as in Fig. 3A) in B to single Intestine 407 cells equilibrated with the CsCl solutions. Hypotonic challenges were made at the arrow in A, and 5–19 min before recordings in B. The zero current level is indicated by the arrowhead (A) or horizontal line (B).
Fig. 9. Effects of arachidonic acid (A–D) and oleic acid (E) on volume-sensitive Cl– currents. The currents were measured by applying alternating pulses (0 to ±40 mV) to single Intestine 407 cells equilibrated with CsCl solutions. The hypotonic challenge was made at the arrow. Upon wash-out of arachidonic acid, bovine serum albumin (BSA, 1 mg/ml) was added in B but not in A. In C, NDGA (10 μM) and indomethacin (10 μM) were added to the hypotonic perfusate before administration of arachidonic acid. In D, polymyxin B was added to the pipette solution. The zero current level is indicated by the arrowhead. The data represent three to nine similar experiments.

Fig. 10. Dose–response curves for effects of arachidonic acid (squares) and oleic acid (circles) on the inward (open symbols) and outward Cl– currents (filled symbols). The currents were observed at ±40 mV in single osmotically swollen Intestine 407 cells equilibrated with the CsCl solutions before and after application of fatty acids. The ordinate represents the percentage of peak currents at maximum inhibition against steady currents before applications of fatty acids. Each symbol represents the mean value of four to eleven experiments with the s.e. of the mean (vertical bar). Since small oil droplets were often found in the bathing solutions containing oleate (but not arachidonate) of ≥10 μM, the effective concentrations of oleic acid would be smaller than those indicated.
Fig. 11. Effects of Cl\(^-\) channel blockers (A) and fatty acids (B) on the volume regulation of single spherical Intestine 407 cells after hypotonic challenges (at the arrow). The relative cell volume was estimated from the diameter measured in the absence (○) and presence of 100 \(\mu\)M SITS (△), 250 \(\mu\)M NPPB (□), 500 \(\mu\)M DPC (◆), 40 \(\mu\)M arachidonic acid (■), 40 \(\mu\)M oleic acid (●) or 40 \(\mu\)M elaidic acid (▲). The effective concentration of oleic acid would be less than 40 \(\mu\)M (see legend for Fig. 10). The data represent three to five similar experiments. The mean cell diameter was 15.7±0.6 \(\mu\)m before osmotic perturbation and 19.8±0.5 \(\mu\)m (23 observations) at peak osmotic swelling.

DISCUSSION

Properties of volume-sensitive Cl\(^-\) channel currents

The volume-sensitive Cl\(^-\) channel in Intestine 407 cells has unique voltage-sensitivities. The instantaneous current shows outward rectification (Fig. 5). The Cl\(^-\) channel current could be maintained in the activated state over the physiological range of membrane potentials but was quickly inactivated at large depolarizations (over +50 mV) (Fig. 4A). Inactivation becomes more rapid with increasing degree of depolarization (Figs 3C and 4A). The Cl\(^-\) current is dependent on the previous conditions inasmuch as greater instantaneous currents were evoked by constant command pulses in the cells to which more negative conditioning pulses had been applied (even after interruption for more than 15 s; Fig. 4E).

The anion selectivity determined from reversal potentials (Fig. 2B, Table 2) was SCN\(^-\) > I\(^-\) > Br\(^-\) > Cl\(^-\) > F\(^-\) > gluconate\(^-\), which corresponds to Eisenman’s sequence I (Wright & Diamond, 1977), suggesting that the anion channel contains weak binding sites. This result is in good agreement with the permeability sequence of volume-sensitive anion currents in a colonic epithelial cell line (Worrell et al. 1989).

Cl\(^-\) transport pathways involved in the regulatory volume decrease were reported to be sensitive to a stilbene-derivative Cl\(^-\) channel blocker (SITS or DIDS) in epithelial MDCK cells (Rothstein & Mack, 1990). In Intestine 407 cells, the volume-sensitive Cl\(^-\) channel was found to show a high sensitivity to SITS (Figs 6 and 7). Recently, a carboxylate analogue Cl\(^-\) channel blocker, 9-anthracencarboxylic acid (9-AC), was found to inhibit Cl\(^-\) pathways activated upon osmotic swelling in guinea-pig jejunal enterocytes (MacLeod & Hamilton, 1991b). Another carboxylate analogue, NPPB, was also found to suppress volume-sensitive Cl\(^-\) currents in a ciliary epithelial cell line (Yantorno et al. 1992). Consistently, NPPB was effective in inhibiting volume-sensitive Cl\(^-\) currents in Intestine 407 cells (Figs 7 and 8).

Hypotonic swelling is known to cause an increase in the cytosolic free Ca\(^{2+}\) concentration in Intestine 407 cells (Hazama & Okada, 1990a, b). Intracellular cyclic AMP increases were also observed in association with osmotic swelling in other cell
species (Watson, 1990; Baquet, Miejer & Hue, 1991). Therefore, there is a possibility that either or both second messengers are involved in activation of volume-sensitive Cl\textsuperscript{−} channels. In Intestine 407 cells, however, swelling-induced Cl\textsuperscript{−} currents were found to be totally independent of cytosolic Ca\textsuperscript{2+} (Fig. 3D) and cyclic AMP. Different types of Cl\textsuperscript{−} currents were activated by increases in cytosolic Ca\textsuperscript{2+} (Fig. 3E) and cyclic AMP, as was the case in a colonic epithelial cell line (Cliff & Frizzell, 1990).

At present, the factors responsible for activation of the Cl\textsuperscript{−} channel are not known. Cell membrane expansion itself might not directly activate the Cl\textsuperscript{−} channel, because the Cl\textsuperscript{−} current activation had a time lag of 5–10 s behind the onset of visible osmotic swelling (Fig. 1).

Direct inhibition by arachidonic acid

In recent years a compelling body of evidence has been amassed that an unsaturated fatty acid, arachidonic acid, which makes up a major component of the cell membrane phospholipids (Irvine, 1982), directly or indirectly modulates ion channels. The present study showed that arachidonic acid inhibits the volume-sensitive Cl\textsuperscript{−} currents (Figs 9 and 10).

There are possibilities that some oxygenase metabolites of arachidonic acid (Needleman et al. 1986) or protein kinase C activated by arachidonic acid (Nishizuka, 1988) mediate the arachidonate effect. However, even in the presence of inhibitors of lipoxygenase, cyclo-oxygenase and protein kinase C, arachidonic acid still blocked the volume-sensitive Cl\textsuperscript{−} current (Fig. 9C and D). Furthermore, another cis unsaturated fatty acid, oleic acid, which is not a substrate for oxidases and ineffective in generating oxygen free radicals (Chan, Chen & Yu, 1988), also blocked the Cl\textsuperscript{−} current (Fig. 9E). Taken together, arachidonic acid appears to inhibit directly the volume-sensitive Cl\textsuperscript{−} channel in Intestine 407 cells.

Comparison with other epithelial chloride channels

Epithelial cells have been known to possess several different kinds of Cl\textsuperscript{−} channels other than the volume-sensitive one. They include (1) the cyclic AMP-activated ohmic Cl\textsuperscript{−} channel with a small single-channel conductance (4–11 pS), identified as the cystic fibrosis transmembrane conductance regulator, CFTR (Anderson, Gregory, Thompson, Sousa, Paul, Mulligan, Smith & Welsh, 1991), (2) the outwardly rectifying Cl\textsuperscript{−} channel with an intermediate single-channel conductance (25–75 pS), which is frequently observed after excision and activated via protein kinase A-mediated phosphorylation (Li, McCann, Liedtke, Nairn, Greengard & Welsh, 1988) and (3) the Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel with a very small single-channel conductance (1–3 pS), which also rectifies in the outward direction (Marty, Tan & Trautmann, 1984; Evans & Marty, 1986; Taleb, Feltz, Bossu & Feltz, 1988; Cliff & Frizzell, 1990).

The present study showed that volume-sensitive Cl\textsuperscript{−} currents rectify in the outward direction and are not activated by Ca\textsuperscript{2+} and cyclic AMP. The single channel conductance was not as yet determined in Intestine 407 cells. However, this was reported to be 23 pS in Ehrlich ascites tumour cells (Hudson & Schultz, 1988) and 75 pS in a colonic epithelial cell line (Worrell et al. 1989).

The anion permeability sequence observed in the present study was similar to that in the outward-rectifying Cl\textsuperscript{−} channel (Halm, Rechkenmer, Schoumacher & Frizzell,
intermediate-conductance,

Further determine phosphorylation excised membranes (Anderson, polarization (Evans & Marty, 1986; Cliff & Frizzell, 1990; Anderson & Welsh, 1991). This sequence is distinct from that reported for the cyclic AMP-activated ohmic Cl\(^-\) channel (Cliff & Frizzell, 1990; Anderson & Welsh, 1991).

The volume-sensitive Cl\(^-\) channel was found to be sensitive not only to a stilbene-derivative Cl\(^-\) channel blocker (SITS) but also to a carboxylate analogue Cl\(^-\) channel blocker (NPPB). A similar sensitivity to stilbene derivatives was observed in the outwardly rectifying Cl\(^-\) channel (Tabcharani, Low, Elie & Hanrahan, 1990; Tilmann, Kunzelmann, Frobe, Cabantchik, Lang, Englert & Greger, 1991) and in the Ca\(^{2+}\)-activated Cl\(^-\) channel (Anderson & Welsh, 1991) but not in the cyclic AMP-activated ohmic Cl\(^-\) channel (Tabcharani et al. 1990; Anderson & Welsh, 1991). Similarly, NPPB blocks the outwardly rectifying Cl\(^-\) channel (Hayslett, Gogelein, Kunzelmann & Greger, 1987; Kunzelmann, Pavenstadt & Greger, 1989; Li et al. 1990; McCann et al. 1989; Giraldez et al. 1989; Sepulveda et al. 1991; Tilmann et al. 1991) but not the cyclic AMP-activated ohmic Cl\(^-\) channel (Champigny, Verrier, Gerard, Mauchamp & Lazdunski, 1990).

As in the outwardly rectifying Cl\(^-\) channel of airway epithelial cells (Anderson & Welsh, 1990; Hwang et al. 1990), volume-sensitive Cl\(^-\) channels in human intestinal epithelial cells were sensitive to arachidonic acid. This is in contrast with small-conductance ohmic Cl\(^-\) channel currents in gastric parietal cells, which are activated by arachidonate (Sakai, Okada, Morii & Takeguchi, 1992).

Volume-sensitive Cl\(^-\) currents exhibit inactivation with time at large depolarizations (Worrell et al. 1989; the present study). Similar voltage-dependent inactivation kinetics was observed in the outwardly rectifying Cl\(^-\) channel current in excised patches (Shoemaker, Frizzell, Dwyer & Farley, 1986; McCann et al. 1989; Tabcharani, Jensen, Riordan & Hanrahan, 1989) and under the whole-cell mode (McCann et al. 1989). In contrast, the Ca\(^{2+}\)-activated Cl\(^-\) channel current shows activation kinetics upon depolarization and inactivation kinetics upon hyperpolarization (Evans & Marty, 1986; Taleb et al. 1988; Cliff & Frizzell, 1990; Anderson & Welsh, 1991; also see Fig. 3E in the present study). Neither marked time-dependent activation nor inactivation was found in the cyclic AMP-activated ohmic Cl\(^-\) channel (Cliff & Frizzell, 1990; Anderson & Welsh, 1991).

On balance, it appears that the properties of volume-sensitive Cl\(^-\) channel currents bear resemblance to those of intermediate-conductance, outwardly rectifying Cl\(^-\) channel currents in many aspects. The outwardly rectifying Cl\(^-\) channel recorded in excised membranes is known to be activated by protein kinase A-mediated phosphorylation (Li et al. 1988). However, volume-sensitive whole-cell Cl\(^-\) currents could be activated even in the presence of an inhibitor of protein kinase A (Fig. 3F). Therefore, there is a possibility that the same channel can be activated by osmotic swelling under the whole-cell mode and by protein kinase A under the excised mode. Further studies by both single-channel and whole-cell recordings are needed to determine the precise relationship between the volume-sensitive Cl\(^-\) channel and the intermediate-conductance, outward rectifier Cl\(^-\) channel.
Physiological relevance

Increases in the Cl− permeability induced by osmotic cell swelling are known to be essential for subsequent volume regulation in a number of cell species (Hoffman & Simonsen, 1989; Okada & Hazama, 1989; Grinstein & Foskett, 1990). In Intestine 407 cells effective blockers of the volume-sensitive Cl− channel such as SITS, NPPB, arachidonic acid and oleic acid were found to abolish the regulatory volume decrease (Fig. 11). In contrast, elaidate and palmitate which did not inhibit the Cl− channel were ineffective in inhibiting the volume regulation. Therefore, it appears that the Cl− channel is volume-regulatory.

Cell volume regulation would be a prerequisite for the physiological function of intestinal epithelial cells, because their vigorous transport activities produce an osmotic gradient across the cell membrane due to active accumulation of osmotically active solutes within the cells (Okada, 1979). A regulatory volume decrease has actually been observed in isolated villus enterocytes after swelling upon Na+−dependent absorption of organic solutes (MacLeod & Hamilton, 1991a).

The physiological significance of Cl− channel modulation by arachidonic acid is not clear. Arachidonic acid and its metabolites are known to be involved in the pathophysiology of several intestinal diseases including intestinal inflammation and anaphylaxis (Powell, 1991). The arachidonic acid-induced inhibition of volume-regulatory Cl− channels might be then at least in part involved in the pathogenesis of these intestinal diseases.

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