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# Phloretin differentially inhibits volume-sensitive and cyclic AMP-activated, but not Ca-activated, $Cl^-$ channels

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**1** Some phenol derivatives are known to block volume-sensitive $Cl^-$ channels. However, effects on the channel of the bisphenol phloretin, which is a known blocker of glucose uniport and anion antiport, have not been examined. In the present study, we investigated the effects of phloretin on volume-sensitive $Cl^-$ channels in comparison with cyclic AMP-activated CFTR $Cl^-$ channels and $Ca^{2+}$-activated $Cl^-$ channels using the whole-cell patch-clamp technique.

**2** Extracellular application of phloretin (over 10 $\mu$M) voltage-independently, and in a concentration-dependent manner ($IC_{50}$ $\sim$30 $\mu$M), inhibited the $Cl^-$ current activated by a hypotonic challenge in human epithelial T84, Intestine 407 cells and mouse mammary C127/CFTR cells.

**3** In contrast, at 30 $\mu$M phloretin failed to inhibit cyclic AMP-activated $Cl^-$ currents in T84 and C127/CFTR cells. Higher concentrations (over 100 $\mu$M) of phloretin, however, partially inhibited the CFTR $Cl^-$ currents in a voltage-dependent manner.

**4** At 30 and 300 $\mu$M, phloretin showed no inhibitory effect on $Ca^{2+}$-dependent $Cl^-$ currents induced by ionomycin in T84 cells.

**5** It is concluded that phloretin preferentially blocks volume-sensitive $Cl^-$ channels at low concentrations (below 100 $\mu$M) and also inhibits cyclic AMP-activated $Cl^-$ channels at higher concentrations, whereas phloretin does not inhibit $Ca^{2+}$-activated $Cl^-$ channels in epithelial cells.
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**Keywords:** Phloretin; volume-sensitive $Cl^-$ channel; $Ca^{2+}$-activated $Cl^-$ channel; cyclic AMP-activated $Cl^-$ channel; cystic fibrosis transmembrane conductance regulator; cell volume regulation

**Abbreviations:** AQP, aquaporin; CFTR, cystic fibrosis transmembrane conductance regulator; dbcAMP, $N^6$,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate; DIDS, dihydro-4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; DMSO, dimethyl sulphoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis($\beta$-aminoethylether)-N,N,N′,N′-tetraacetic acid; HEPES, N-(hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid); IBMX, 3-isobutyl-1-methylxanthine; NMDG, N-methyl-D-glucamine; RVD, regulatory volume decrease; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid; TRIS, tris(hydroxymethyl)aminomethane

## Introduction

Cell volume regulation is of fundamental importance to mammalian cells under anisotonic conditions, in maintaining cell structural integrity and a variety of other cellular functions (see Lang, 1998; Okada, 1998). In response to a hypotonic challenge, cells swell and then gradually recover their original volume. This process, known as regulatory volume decrease (RVD), involves the efflux of $K^+$ and $Cl^-$ as well as organic solutes including some amino acids (Strange *et al*., 1996; Okada, 1997). As intracellular solutes are lost, water egress follows passively according to the osmotic gradient across the cell membrane. Different pathways have been known to be involved in the volume-regulatory efflux of KCl in different cell types (Hoffmann & Simonsen, 1989; Okada, 1997). Over the past 10 years a volume-sensitive $Cl^-$ channel, thought to be the principal anion channel involved in RVD, has been extensively studied, and its electrophysiological characteristics (such as outward rectification and voltage-dependent inactivation), thoroughly described (Strange *et al*., 1996; Nilius *et al*., 1997; Okada, 1997). However, its molecular identity and activation mechanism remain unclear (Okada, 1997; Okada *et al*., 1998), although the ClC-3 gene of the ClC family of $Cl^-$ channels was reported to encode a type of swelling-activated $Cl^-$ channel (Duan *et al*., 1997). Cloning of the gene encoding the volume-sensitive $Cl^-$ channel has been quite difficult, because most cell types express this channel endogenously, thereby hindering the expression cloning. Moreover, overexpression of candidate genes or proteins often upregulates the activity of endogenous anion channels (Voets *et al*., 1996; Okada, 1997). Therefore, it is very important to search for a highly specific blocker useful for the protein purification.

In epithelial cells, there exist at least three major types of anion channel: volume-sensitive, cyclic AMP-activated and

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$Ca^{2+}$-activated $Cl^-$ channels. The cystic fibrosis transmembrane conductance regulator (CFTR), which is the product of the cystic fibrosis gene, is known to be a multifunctional protein that functions not only as a cyclic AMP-activated epithelial $Cl^-$ channel but also as a regulator of other channels and transporters (Kunzelmann & Schreiber, 1999; Schwiebert *et al*., 1999). In cardiac myocytes, activation of CFTR under stimulation of $\beta$-adrenergic receptors was reported to be responsible for $Cl^-$ efflux during the RVD (Wang *et al*., 1997). However, it has not been precisely determined which is the main anionic pathway for RVD under physiological conditions in CFTR-expressing epithelial cells, CFTR or the volume-sensitive $Cl^-$ channel. In many cell types, an increase in intracellular free $Ca^{2+}$ has been observed under hypotonic conditions (Hazama & Okada, 1990; McCarty & O'Neil, 1992). Although it is well known that strongly outward-rectifying $Cl^-$ currents can be activated by elevation of intracellular $Ca^{2+}$ concentration in many cell types to date, little is known about the involvement of the $Ca^{2+}$-activated $Cl^-$ channel in RVD. In $Cl^-$-secreting epithelial cells both CFTR and $Ca^{2+}$-activated $Cl^-$ channels are known to operate in concert for $Cl^-$ and fluid secretion, and a $Ca^{2+}$-activated secretory pathway for $Cl^-$ was shown to substitute for CFTR in various tissues of cystic fibrosis patients and CFTR-knockout mice (Wagner *et al*., 1992; Rozmahel *et al*., 1996). However, it is not known whether volume-sensitive $Cl^-$ channels are involved in epithelial anion secretion under physiological or pathological conditions. Thus, the discovery of selective blockers would make available pharmacological tools that would significantly enhance the investigation of the function and molecular basis of $Cl^-$ channels.

A wide variety of $Cl^-$ channel blockers are known to block volume-sensitive $Cl^-$ channels, but no specific blocker for volume-sensitive $Cl^-$ channels has been found so far (Nilius *et al*., 1997; Okada, 1997). Among them, some phenol-derivatives, including gossypol (Gschwentner *et al*., 1996; Szücs *et al*., 1996), 2-aminomethyl-4-t-butyl-6-iodophenol hydrochloride (Simchowitz *et al*., 1993), niflumic acid or flufenamic acid (see Nilius *et al*., 1997; Okada, 1997), and nordihydroguaiaretic acid (Doroshenko & Neher, 1992; Fatherazi *et al*., 1994), were found to be the members of a family of volume-sensitive $Cl^-$ channel blockers (Roy *et al*., 1998). Also, not only gossypol (Strange *et al*., 1993; McManus *et al*., 1994) but also a bisphenol derivative phloretin (Reeves & Cammarata, 1996) were found to block swelling-induced efflux of inositol or myo-inositol. Although there is some debate whether volume-sensitive organic osmolyte transport is mediated by volume-sensitive $Cl^-$ channels or separate channels (Lambert & Hoffmann, 1994; Morán *et al*., 1997; Stutzin *et al*., 1999; Shennan & Thomson, 2000, Stegen *et al*., 2000), there exists a possibility that phloretin is a potent blocker of volume-sensitive $Cl^-$ channels. Phloretin was shown to have no effect on the $Ca^{2+}$-activated $Cl^-$ conductance in T84 cells at 50 $\mu$M (Worrell & Frizzell, 1991) and on the cyclic AMP-activated $Cl^-$ current in CFTR-expressing *Xenopus* oocytes at 350 $\mu$M (Schreiber *et al*., 1997). However, whether phloretin inhibits the volume-sensitive $Cl^-$ channel has not been examined.

In the present study, we investigated the effects of phloretin on volume-sensitive $Cl^-$ channels as well as cyclic AMP-activated and $Ca^{2+}$-activated $Cl^-$ channels in human colonic T84 cells, which are known to express these three types of $Cl^-$ channels (Worrell *et al*., 1989; Cliff & Frizzell, 1990; Chang *et al*., 1992; Merlin *et al*., 1998), using the whole-cell patch-clamp technique. Since there is the possibility that phloretin exhibits different effects on these channels in different cell types, two additional cell lines were employed: human epithelial Intestine 407 cells, in which properties of volume-sensitive $Cl^-$ channels have been well characterized (Okada, 1997), and mouse mammary C127 cells stably transfected with cDNA for CFTR (C127/CFTR cells), which express not only cyclic AMP-activated $Cl^-$ channels but also volume-sensitive $Cl^-$ channels (Hazama *et al*., 2000). Here, phloretin was found to block preferentially volume-sensitive $Cl^-$ channels at low concentrations. A preliminary account of these results has appeared in abstract form (Fan *et al*., 1999).

## Methods

### *Cell culture*

T84 cells were obtained from the American Type Culture Collection (ATCC No. CCL-248, Rockville, MD, U.S.A.) and grown at 37°C in a 25 $cm^2$ flask gassed with 95% air and 5% $CO_2$. The monolayers were cultured in a medium composed of a 95% 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium supplemented with 2.5 mM L-glutamine and 5% foetal bovine serum, as reported previously (Arreola *et al*., 1995). For patch-clamp studies, cells on glass slips were provided 1–3 days after plating.

A human epithelial cell line, Intestine 407, was cultured in monolayer in Fischer medium supplemented with 10% newborn calf serum under a 95% air-5% $CO_2$ atmosphere, as described previously (Kubo & Okada, 1992). Before patch-clamp experiments, the monolayer of cells was isolated to single spherical cells by pipetting and was cultured in suspension with agitation for 15–240 min.

A stable transfectant of the mouse mammary tumour cell line, C127i, with the cDNA for human CFTR (C127/CFTR) was the kind gift of Dr M.J. Welsh (University of Iowa). C127/CFTR cells were cultured in monolayer in Dulbecco's modified Eagle's medium (DMEM, High Glucose) with 10% foetal bovine serum (FBS) and 200 $\mu$g $ml^{-1}$ geneticin. Single cells were prepared by washing the monolayer with $Ca^{2+}$- and $Mg^{2+}$-free Hanks' balanced salt solution ($Ca^{2+}$-, $Mg^{2+}$-free HBSS) twice then incubating with 0.25% trypsin-EDTA solution, and cultured in suspension with agitation for 10–180 min before use in patch-clamp experiments.

### *Patch-clamp whole-cell recordings*

Whole-cell recordings were performed, as reported previously (Kubo & Okada, 1992; Zhou *et al*., 1998). Wide-tipped electrodes (around 2 MΩ) were fabricated from borosilicate glass capillaries using a Brown-Flaming Micropipette Puller (Model P-97, Sutter Instrument Co., Novato, CA, U.S.A.). The series resistance ($\leqslant$5 MΩ) was compensated (about 75%) to minimize voltage errors in experiments on volume-sensitive $Cl^-$ currents. A variety of voltage-pulse protocols, using both square-shaped and ramped voltage pulses, were used to examine the $Cl^-$ currents. Details of the voltage-pulse protocols are provided in the appropriate figure legends.

Currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster, CA, U.S.A.). Current signals were filtered at 0.5–1 kHz using a 4-pole Bessel-filter and digitized at 2–4 kHz. pCLAMP software (version 6.0.2; Axon Instruments) and Origin 5.0 (Microcal Software Inc., Northampton, MA, U.S.A.) were used for command control, data acquisition and analysis. The amplitude of instantaneous current was measured at 2 ms after the step pulse onset. By fitting the time course of inactivation to a double exponential function, the steady-state current was evaluated, and the inactivation kinetics was analysed.

Bath solution was maintained at 37°C in experiments on cyclic AMP-activated $Cl^-$ currents in T84 cells. All other experiments were carried out at room temperature (23–26°C). Data are given as the mean ± s.e.mean. Statistical differences of the data were evaluated by Student's *t*-test and were considered significant at $P<0.05$.

### *Solutions and chemicals*

For experiments on volume-sensitive $Cl^-$ currents, isotonic bath solution (osmolality 330 mosmol $kg{\cdot}H_2O^{-1}$) contained (in mM) CsCl 110, $MgSO_4$ 5, mannitol 100, HEPES 12, and TRIS 8 (adjusted to pH 7.4 with $H_2SO_4$). Hypotonic bath solution (osmolality 270 mosmol $kg{\cdot}H_2O^{-1}$) was prepared by reducing mannitol to 40 mM. In some experiments, the CsCl concentration was reduced to 11 mM by replacing with mannitol. Pipette solution (osmolality 300 mosmol $kg{\cdot}H_2O^{-1}$) contained (in mM) CsCl 110, $MgSO_4$ 2, EGTA 1, $Na_2$-ATP 1, mannitol 50, and Na-HEPES 15 (adjusted to pH 7.4 with $H_2SO_4$). For the experiments on cyclic AMP-activated $Cl^-$ currents in C127/CFTR cells, the osmolality of isotonic bath solution was adjusted to 310 mosmol $kg{\cdot}H_2O^{-1}$ by reducing mannitol to 80 mM, and the concentrations of $Na_2$-ATP and mannitol in the pipette solution were changed to 2 mM and 47 mM, respectively.

The solutions employed in experiments on cyclic AMP-activated $Cl^-$ currents in T84 cells were as those described previously (Cliff & Frizzell, 1990). Bath solution contained (in mM): NaCl 115, *N*-methyl-D-glucamine (NMDG) glutamate 40, potassium glutamate 5, $MgCl_2$ 2, $CaCl_2$ 1, and HEPES/NaOH 5 (pH 7.3, osmolality 330 mosmol $kg{\cdot}H_2O^{-1}$). Pipette solution (osmolality 300 mosmol $kg{\cdot}H_2O^{-1}$) contained (in mM): KCl 115, NMDG glutamate 25, EGTA 0.5, $CaCl_2$ 0.19, $MgCl_2$ 2, $Na_2ATP$ 2, $Na_3GTP$ 0.05, and HEPES/KOH 5 (pH 7.3). $Na^+$- and $K^+$-free

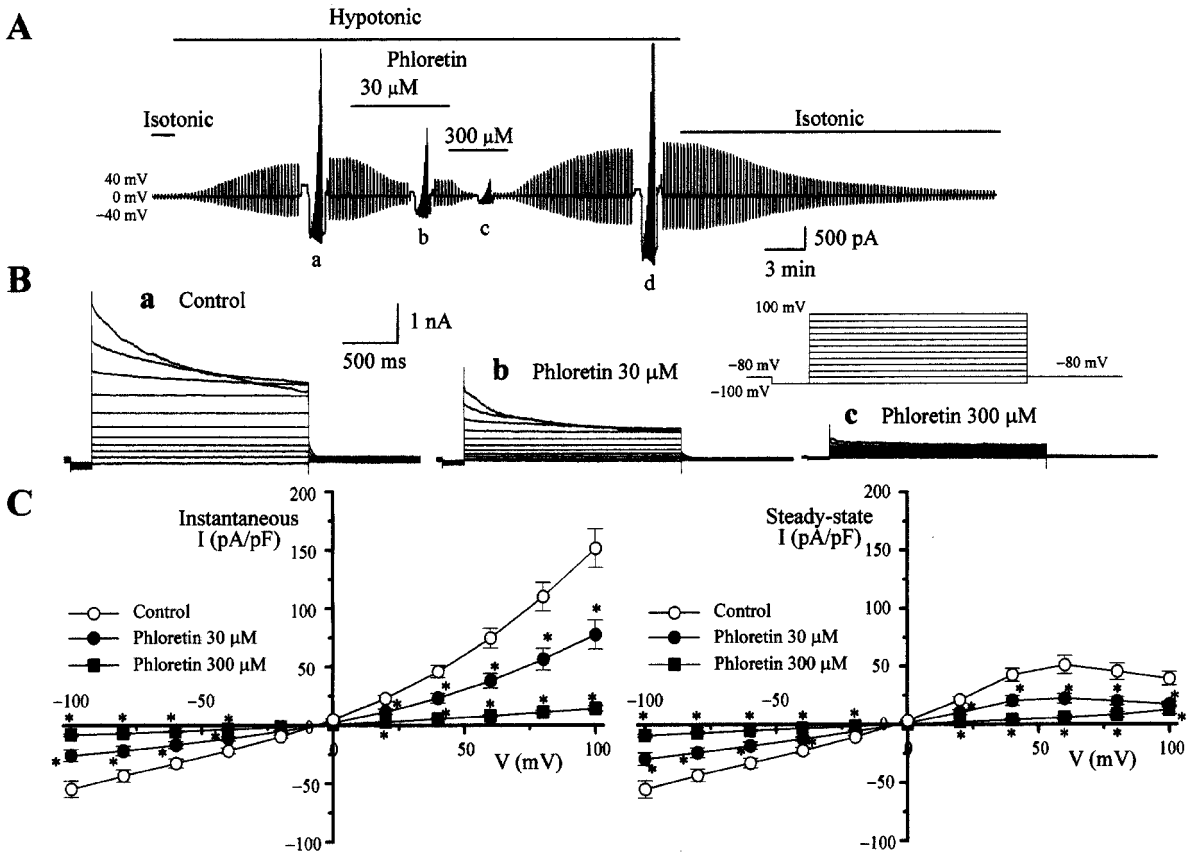


**Figure 1** Effects of extracellular phloretin on volume-sensitive whole-cell $Cl^-$ currents in T84 cells. (A) typical record before and after osmotic cell swelling in the absence or presence of phloretin at different concentrations (indicated by horizontal lines) in bath solution during application of alternating pulses (2-s duration, every 15 s) from 0 mV to ±40 mV or of step pulses (2-s duration) from a pre-potential at −100 mV (0.2-s duration) to test potentials of −100 mV to +100 mV in 20-mV increments (at a, b, c & d). (B) expanded traces of current responses (a, b & c in A) to step pulses in the absence (a) or presence of phloretin at 30 μM (b) or 300 μM (c). Inset: the step pulse protocol. (C) The instantaneous (left) and steady-state (right) current-voltage relationships of volume-sensitive $Cl^-$ currents in the absence (control) or presence of phloretin at 30 μM or 300 μM. Each symbol represents the mean current (with the s.e.mean: vertical bars) of 8 to 12 experiments. *Significantly different from control values.

solutions containing $NMDG^+$ were prepared by replacing NaCl and KCl with NMDG-Cl in the above bath and pipette solutions, respectively, and were used for the experiments on $Ca^{2+}$-activated $Cl^-$ currents in T84 cells. In some experiments, a part of the $Cl^-$ content was replaced with glutamate to change the equilibrium potential for $Cl^-$ ions and details are provided in the Results section.

The composition of $Ca^{2+}$-, $Mg^{2+}$-free HBSS was (in mM): KCl 5.36, $KH_2PO_4$ 0.441, NaCl 136.7, $NaHCO_3$ 4.17, $Na_2HPO_4$ 0.385, and D-glucose 5.55 (pH 7.3). Trypsin-EDTA solution was: 113 mM $Na_2HPO_4$, 1.69 mM $KH_2PO_4$, 0.2% EDTA, and 0.25% trypsin (pH 7.3).

Forskolin, $N^6$,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (dbcAMP) and calphostin C were obtained from Wako Chemicals (Osaka, Japan), and other chemicals were from Sigma (St. Louis, MO, U.S.A.). Stock solutions of phloretin, calphostin C and 3-isobutyl-1-methylxanthine (IBMX) in dimethyl sulphoxide (DMSO) and those of forskolin, ionomycin and phloridzin in ethanol were diluted to the desired final concentrations just before use. The concentration of DMSO or ethanol never exceeded 0.1% and had no effect on whole-cell currents.

## Results

### *Effects of phloretin and phloridzin on volume-sensitive $Cl^-$ currents*

Basal whole-cell currents recorded in T84 cells under isotonic conditions were extremely small and never affected by extracellular application of phloretin (data not shown, $n=4$). Upon exposure to 82% hypotonic bath solution, activation of outwardly rectifying currents (Figure 1A) that exhibited time-dependent inactivation at large positive potentials (Figure 1B), was observed in T84 cells, as reported originally by Worrell *et al.* (1989). When the bath $Cl^-$ concentration was reduced from 110 mM to 11 mM, the reversal potential ($E_{rev}$) was shifted by $41.4\pm3.6$ mV ($n=4$), confirming that these currents were largely anion-selective. The volume-sensitive $Cl^-$ current was markedly suppressed by 30 $\mu$M phloretin and almost completely by 300 $\mu$M phloretin (Figure 1A). The effect of phloretin was rapid in onset and reversible. The current-voltage (I–V) curves for instantaneous (left panel) and steady-state currents (right panel) are shown in Figure 1C. There was no obvious voltage dependence of the inhibitory effect of phloretin over the entire voltage range examined.

The time course of inactivation of volume-sensitive $Cl^-$ currents observed upon large depolarization could be fitted by a double exponential function in T84 cells (Figure 2A), as reported previously (Levitan & Garber, 1995). Neither the fast ($\tau_f$) nor slow time constant ($\tau_s$) was significantly affected by 30 $\mu$M phloretin (Figure 2B).

The inhibitory effect of phloretin was further investigated in Intestine 407 cells, which lack CFTR mRNA (Hazama *et al.*, 1998) and a $Ca^{2+}$-activated $Cl^-$ conductance (Hazama & Okada, 1988; Tilly *et al.*, 1994). Extracellular phloretin similarly suppressed the volume-sensitive $Cl^-$ current in Intestine 407 cells in a concentration-dependent manner (Figure 3A). As observed in T84 cells, inactivation kinetics at large positive potentials were not affected by phloretin

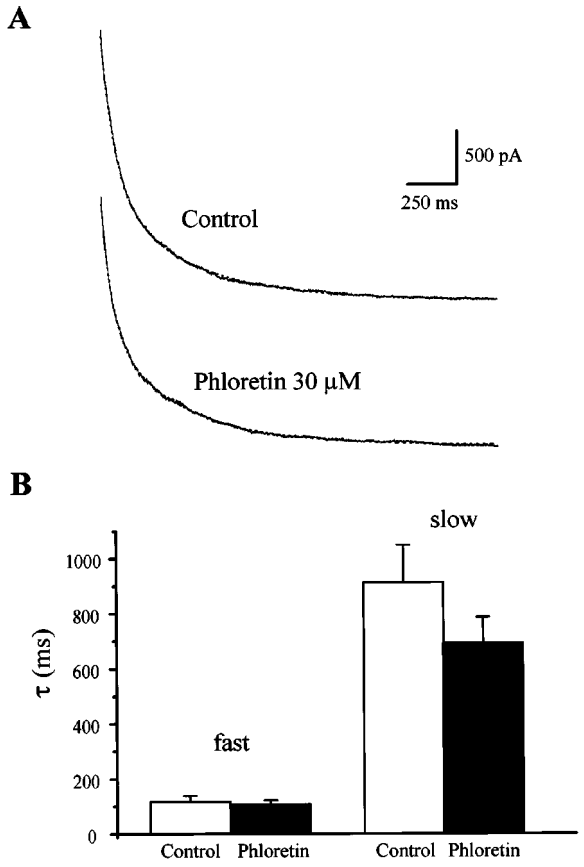


**Figure 2** Effects of phloretin on depolarization-induced inactivation kinetics of volume-sensitive $Cl^-$ currents in T84 cells. (A) inactivation time courses during application of steps (2-s duration) to +100 mV in the absence (control) or presence of 30 $\mu$M phloretin. Dotted curves are biexponential fits. (B) fast ($\tau_f$) and slow ($\tau_s$) time constants for inactivation of volume-sensitive $Cl^-$ currents in the absence (control) or presence of 30 $\mu$M phloretin at +100 mV (means±s.e.mean, $n=8$). Differences between values of control experiments and those with phloretin are not statistically significant.

(data not shown, $n=11$). Inhibiting effects on both instantaneous and steady-state currents were also voltage-independent in Intestine 407 cells (Figure 3A).

Outwardly rectifying $Cl^-$ currents could also be activated by hypotonic stress in C127/CFTR cells, as reported previously (Krouse *et al.*, 1994; Xia *et al.*, 1996; Hazama *et al.*, 2000). Reduction of bath $Cl^-$ from 110 mM to 11 mM induced a shift of $E_{rev}$ by $40.1\pm3.2$ mV ($n=3$). A reversible inhibitory effect of extracellular phloretin on the volume-sensitive $Cl^-$ current was also observed in C127/CFTR cells (Figure 3B). The inactivation kinetics at large positive potentials were not affected by phloretin (data not shown, $n=10$). The I–V relationships show that the effects of phloretin on instantaneous and steady-state currents were also voltage-independent (Figure 3B).

Figure 4 shows concentration-inhibition curves for phloretin effects on volume-sensitive $Cl^-$ currents recorded at −60 mV (A) and +60 mV (B) in the three cell lines. The $IC_{50}$ values estimated by curve-fitting were $26.1\pm6.0$ and $31.2\pm3.4$ $\mu$M ($n=7-11$) in Intestine 407 cells, $24.9\pm2.3$ and $27.8\pm2.3$ $\mu$M ($n=6-8$) in C127/CFTR cells, and $36.4\pm6.0$

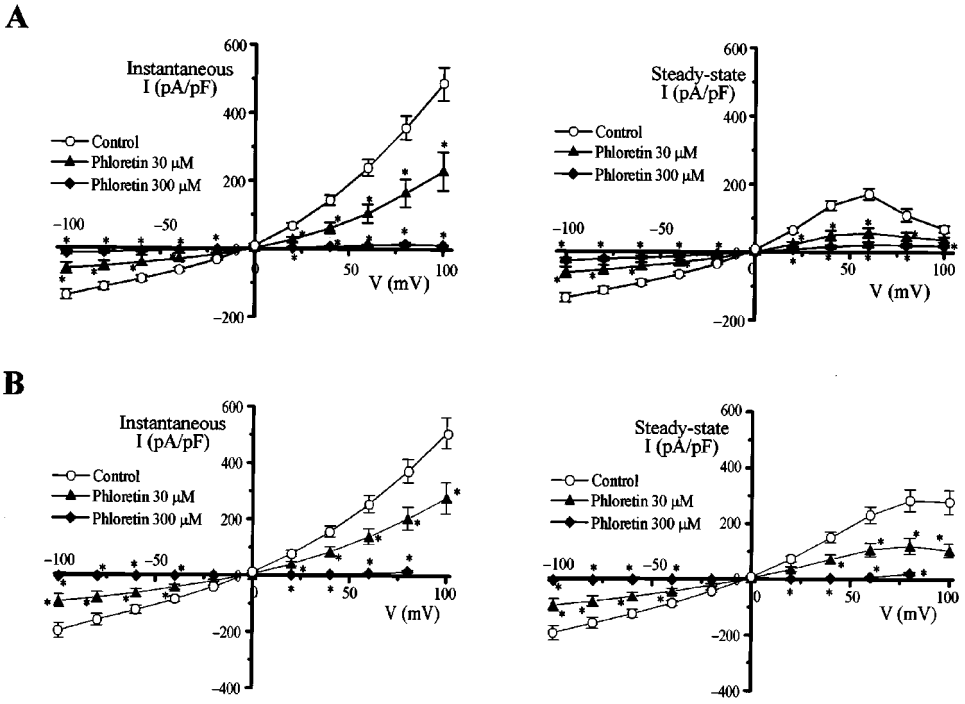


**Figure 3** Effects of extracellular phloretin on volume-sensitive whole-cell $Cl^-$ currents in Intestine 407 (A) and C127/CFTR cells (B). The instantaneous (left) and steady-state (right) current-voltage relationships were observed by applying step pulses (the protocol given in Figure 1B: inset) in the absence (control) or presence of phloretin at 30 $\mu$M or 300 $\mu$M. Each symbol represents the mean current (with the s.e. mean: vertical bars) of seven to 11 experiments. *Significantly different from control values.

and $42.3 \pm 7.6$ $\mu$M ($n = 6-16$) in T84 cells at $-60$ and $+60$ mV, respectively. There was no significant difference between the three cell lines in the $IC_{50}$ values at either voltage. Neither was there any significant difference between the voltages in the $IC_{50}$ values obtained in any of the cell lines.

In contrast to phloretin, extracellular phloridzin (100 $\mu$M), a glucoside of phloretin, showed no effect on the volume-sensitive $Cl^-$ current in Intestine 407 cells (Figure 5A). Inactivation kinetics (B) and I–V relations (C) were not affected by phloridzin. No significant effect of phloridzin was observed even at a concentration as high as 1 mM; either in Intestine 407 or T84 cells (data not shown, $n = 3$ each).

When incorporated in the pipette (intracellular) solution, phloretin failed to inhibit the volume-sensitive $Cl^-$ current in T84, Intestine 407 and C127/CFTR cells even at a high concentration (300 $\mu$M) (data not shown, $n = 5-24$). Both instantaneous and steady-state currents were little affected by 300 $\mu$M phloretin, when applied from the intracellular side. Thus, it is likely that the action site of phloretin is located on the extracellular side. Protein kinase C (PKC) is known to be inhibited not only by phloretin (Gschwendt *et al.*, 1984) but also by phloridzin (Shoji *et al.*, 1997). Therefore, it is unlikely that the phloretin effect on volume-sensitive $Cl^-$ currents was mediated by inhibition of PKC. Actually, extracellular application of calphostin C (500 nM), which is a specific inhibitor of PKC with an $IC_{50}$ of 50 nM (Kobayashi *et al.*, 1989), did not affect the amplitude, the inactivation kinetics and the I–V relations of volume-sensitive $Cl^-$ currents in Intestine 407 cells (data not shown, $n = 7$).

### *Effects of phloretin on $Ca^{2+}$-activated $Cl^-$ currents*

Outwardly rectifying whole-cell currents were activated transiently by adding the $Ca^{2+}$ ionophore, ionomycin (1–2.5 $\mu$M), to isotonic bath solution containing 1 mM $Ca^{2+}$ in T84 cells (Figure 6A), as reported previously (Cliff & Frizzell, 1990; Merlin *et al.*, 1998). However, neither Intestine 407 nor C127/CFTR cells responded with current activation to ionomycin under the experimental conditions (data not shown, $n = 12$ or 5). In T84 cells, the current responses to step pulses exhibited time-dependent activation at positive potentials and inactivation at negative potentials (Figure 6Ba). When the bath $Cl^-$ concentration was reduced from 121 mM to 19 mM, the reversal potential was shifted by $31.1 \pm 0.9$ mV ($n = 5$), demonstrating the anion selective nature of the current. After reaching the peak response, ionomycin-activated currents gradually decreased and soon reached either a basal level (Figure 6A, left trace) or a plateau level larger than the basal level (Figure 6A, right trace; Figure 6Bb). When 30 $\mu$M phloretin was applied to the bath solution after ionomycin-induced currents had attained the steady-state level, the current was never suppressed but consistently increased (Figure 6A, right trace). The reactivated current also exhibited outward rectification as well as voltage-dependent inactivation and activation kinetics (Figure 6Bc). Reduction of the bath $Cl^-$ concentration from 121 mM to 19 mM caused a shift of the reversal potential by $31.1 \pm 0.8$ mV ($n = 6$) in the presence of 30 $\mu$M phloretin. Essentially the same results were obtained with 300 $\mu$M phloretin (data not shown, $n = 2$).

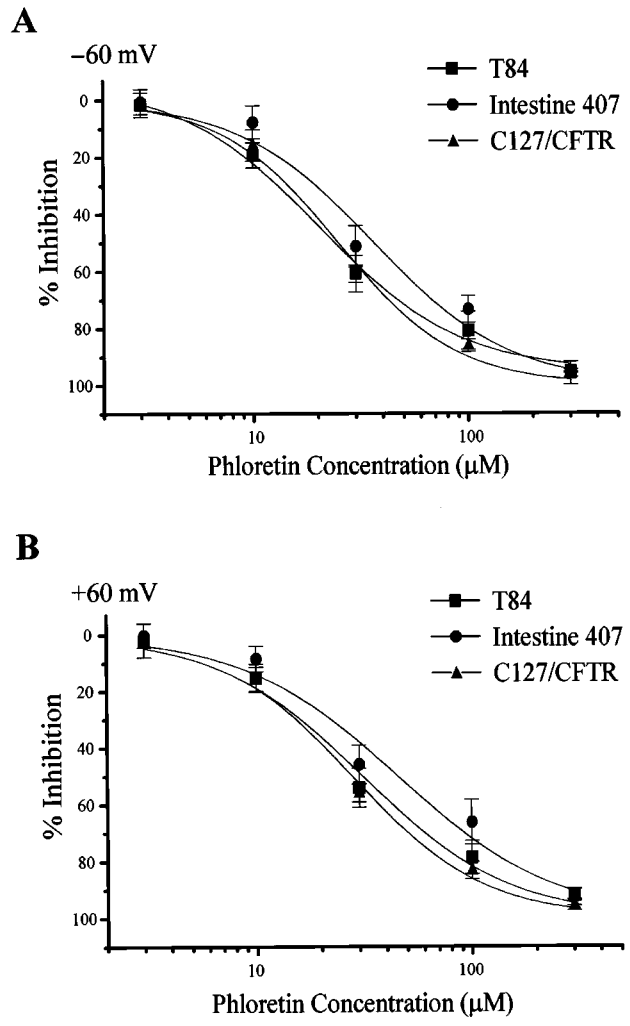


**Figure 4** Concentration-inhibition curves for phloretin-induced inhibition of volume-sensitive $Cl^-$ currents recorded at −60 mV (A) and +60 mV (B) in T84, Intestine 407 and C127/CFTR cells. Each symbol represents the mean±s.e.mean ($n$=6–16). Data were fitted to a sigmoidal relation with the $IC_{50}$ values given in the text and with a Hill coefficient ($h$) of 1.4, 1.4 and 1.6 at −60 mV as well as 1.2, 1.3 and 1.4 at +60 mV for T84, Intestine 407 and C127/CFTR cells, respectively.

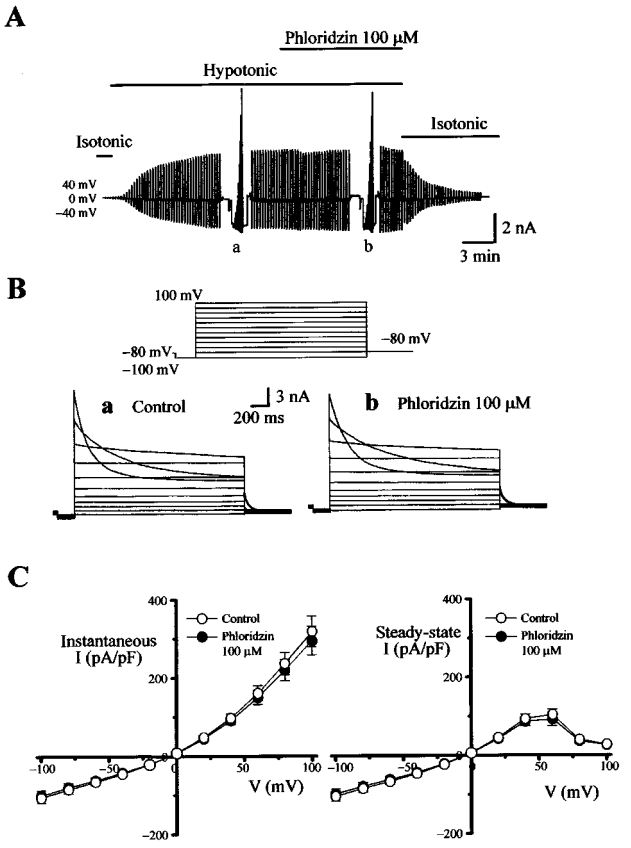


**Figure 5** Effects of extracellular phloridzin on whole-cell volume-sensitive $Cl^-$ currents in Intestine 407 cells. (A) typical record before and after osmotic cell swelling in the absence or presence of 100 μM phloridzin (indicated by horizontal lines) in bath solution during the application of alternating pulses from 0 mV to ±40 mV or of step pulses from −100 mV to +100 mV in 20-mV increments. Gain of chart recorder was changed by one-half during applying step pulses at a and b. (B) expanded traces of current responses (a & b in A) to step pulses in the absence (a) or presence of phloridzin at 100 μM (b). Inset: the step pulse protocol. (C) Instantaneous and steady-state current-voltage curves measured in the absence (control) or presence of 100 μM phloridzin. Differences between values of control experiments and those with phloridzin are not statistically significant.

In the absence of ionomycin, no current was activated by 30 μM phloretin added to the bath solution containing 1 mM $Ca^{2+}$ in T84 cells, but prominent current activation was induced by ionomycin in the presence of phloretin (Figure 6C). Ionomycin-induced currents were sustained for a longer time in the presence of phloretin (Figure 6C) than in its absence (Figure 6A). However, the peak amplitude of ionomycin-induced currents in the presence of 30 μM phloretin was not different from that in its absence (Figure 6D).

*Effects of phloretin on cyclic AMP-activated $Cl^-$ currents*

A cocktail of forskolin (10 μM), dbcAMP (500 μM) and IBMX (400 μM) failed to induce activation of $Cl^-$ currents in Intestine 407 cells (data not shown, $n$=5). However, the cyclic AMP stimulation induced activation of currents in a reversible manner in T84 cells (Figure 7A), as reported previously (Cliff & Frizzell, 1990; Chang *et al*., 1992; Merlin *et al*., 1998). The current was activated at the equilibrium potential to $K^+$ ($E_K$ = −84 mV), whereas no current activation was observed at $E_{Cl}$ (=0 mV). Current responses to step pulses exhibited little time-dependent activation and inactivation (Figure 7Bb). The I–V curve showed a nearly linear relationship in the voltage range between −100 and +100 mV, and the reversal potential was close to $E_{Cl}$ (0 mV) (Figure 7C: open circles). The reversal potential was shifted by 45.8±4.4 mV ($n$=4), when the bath $Cl^-$ concentration was reduced from 110 mM to 11 mM.

No effect of phloretin was observed at 30 μM on the cyclic AMP-activated CFTR current (see Figure 7D). However, when the phloretin concentration was increased to 300 μM, the inward component of cyclic AMP-activated $Cl^-$ currents was partially inhibited, without producing significant inhibition of the outward component (Figure 7Bb; Figure 7C: filled circles).

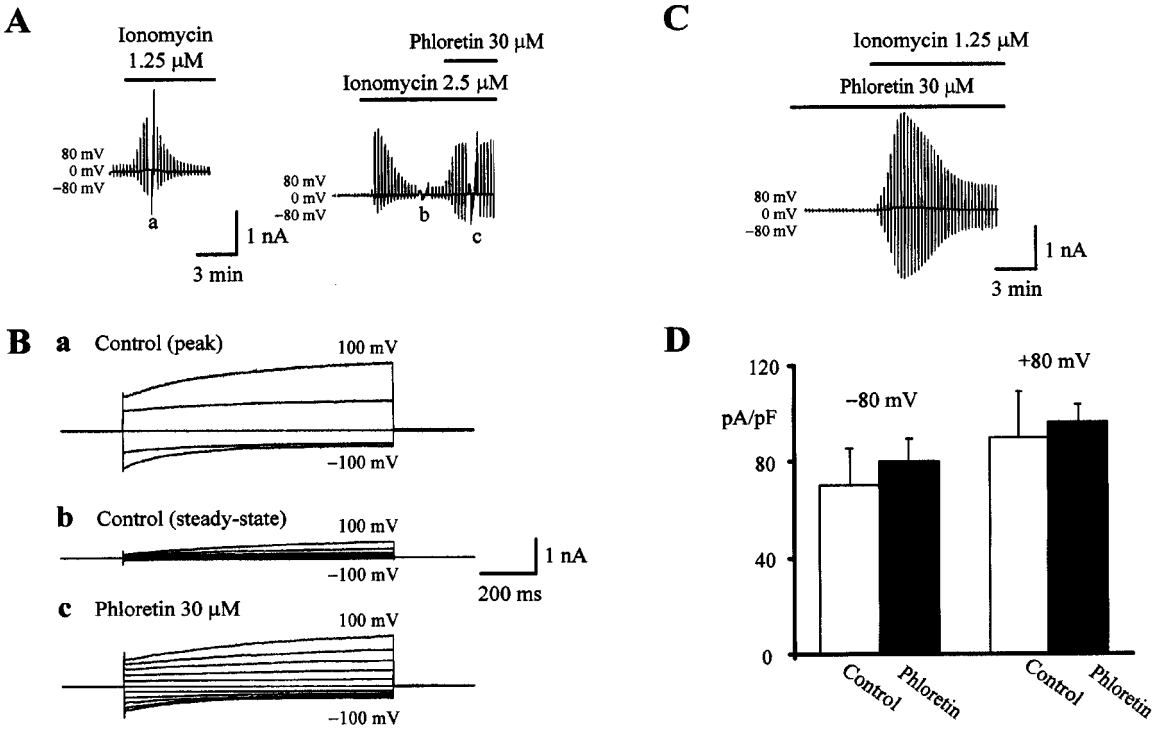


**Figure 6** Effects of extracellular phloretin on $Ca^{2+}$-activated whole-cell $Cl^-$ currents in T84 cells. (A) Typical record of the ionomycin-induced whole-cell $Cl^-$ currents in the absence or presence of 30 $\mu$M phloretin (indicated by horizontal lines) in bath solution during application of ramp pulses from 0 mV to $\pm$80 mV or of step pulses (1-s duration) from $-$100 mV to $+$100 mV in 20- or 50-mV increments (at a, b & c). (B) Expanded traces of current responses (a, b & c in A) to step pulses in the absence (a, b) or presence of phloretin at 30 $\mu$M (c). (C) Representative record of ionomycin-induced activation of $Ca^{2+}$-dependent whole-cell $Cl^-$ currents after preincubating with 30 $\mu$M phloretin during application of ramp pulses from 0 mV to $\pm$80 mV. (D) Effects of phloretin on the current density at $-$80 mV or $+$80 mV. Data were shown by the mean$\pm$s.e.mean ($n$=7–8). Differences between values of control and those with phloretin are statistically insignificant.

Figure 7D shows concentration-inhibition curves for phloretin effects on cyclic AMP-activated $Cl^-$ currents in T84 cells. At $-$60 mV and $+$60 mV, respectively, the $IC_{50}$ values were 252.0$\pm$20.7 $\mu$M and 474.7$\pm$24.5 $\mu$M ($n$=7–13) in T84 cells. A similar voltage-dependent effect of phloretin was also observed in C127/CFTR cells, and the $IC_{50}$ values were 194.7$\pm$37.3 $\mu$M and 355.1$\pm$37.1 $\mu$M ($n$=8–10). The values at $-$60 mV are significantly smaller than those at $+$60 mV. All these $IC_{50}$ values for CFTR $Cl^-$ currents were one order of magnitude greater than those for volume-sensitive $Cl^-$ currents (Figure 4).

On the other hand, no effect on cyclic AMP-activated currents was induced by intracellular application of 300 $\mu$M phloretin in T84 cells (data not shown, $n$=9).

In C127/CFTR cells, whole-cell CFTR $Cl^-$ currents could also be activated by the cocktail of forskolin (10 $\mu$M), dbcAMP (500 $\mu$M) and IBMX (400 $\mu$M), as reported previously (Hazama *et al*., 2000). Extracellular application of 30 $\mu$M phloretin showed no effect on either the inward or outward components of cyclic AMP-activated $Cl^-$ currents at $-$100 and $+$100 mV (data not shown, $n$=8). However, 300 $\mu$M phloretin, although only in part, suppressed preferentially the outward component of cyclic AMP-activated $Cl^-$ currents in C127/CFTR cells (data not shown, $n$=9).

In contrast, extracellular application of a PKC inhibitor, calphostin C (500 nM), failed to affect cyclic AMP-induced currents in C127/CFTR cells (data not shown, $n$=5).

## Discussion

Phloretin is the aglucon of phloridzin, a sap-soluble pigment extracted from the root bark of apple trees (Seshadri, 1951), which is known to inhibit the $Na^+$-independent glucose transporter (glucose uniporter: GLUT) (Sahagian, 1965; Betz *et al*., 1975) with an $IC_{50}$ of 50–60 $\mu$M (Kasahara & Kasahara, 1996) as well as protein kinase C (Gschwendt *et al*., 1984). Moreover, this compound is known to inhibit anion exchange in human red blood cells at 1–3 $\mu$M (Fröhlich & Gunn, 1987), the aquaporin-3 (AQP3) water channel and urea transporter at 100–350 $\mu$M (Ishibashi *et al*., 1994; Echevarria *et al*., 1996), promiscuous solute channel, AQP9, at 100 $\mu$M (Tsukaguchi *et al*., 1998), $Na^+$-dependent dicarboxylate transporter, SDCT1, at 500 $\mu$M (Chen *et al*., 1998) and swelling-induced taurine release at 100 $\mu$M (Hall, 1995) as well as myo-inositol release at 200 $\mu$M (Reeves & Cammarata, 1996). Phloretin was also found to suppress several types of voltage-gated cation channels: $Ca^{2+}$ channels in pituitary adenoma cells at 250–300 $\mu$M (Prevarskaya *et al*., 1994), $K^+$ channels in nerve fibers at 10–100 $\mu$M (Strichartz *et al*., 1980; Klusemann & Meves, 1991; 1992) and $Na^+$ channels in PC12 cells at 300 $\mu$M (S. Morishima & Y. Okada, 2001, unpublished observations). In contrast, phloretin (10–200 $\mu$M) is known to enhance endogenous $Ca^{2+}$-activated $K^+$ channels in several cell types (Koh *et al*., 1994; Shin *et al*., 1996; Bringmann & Reichenbach, 1997) and the cloned *Slo*

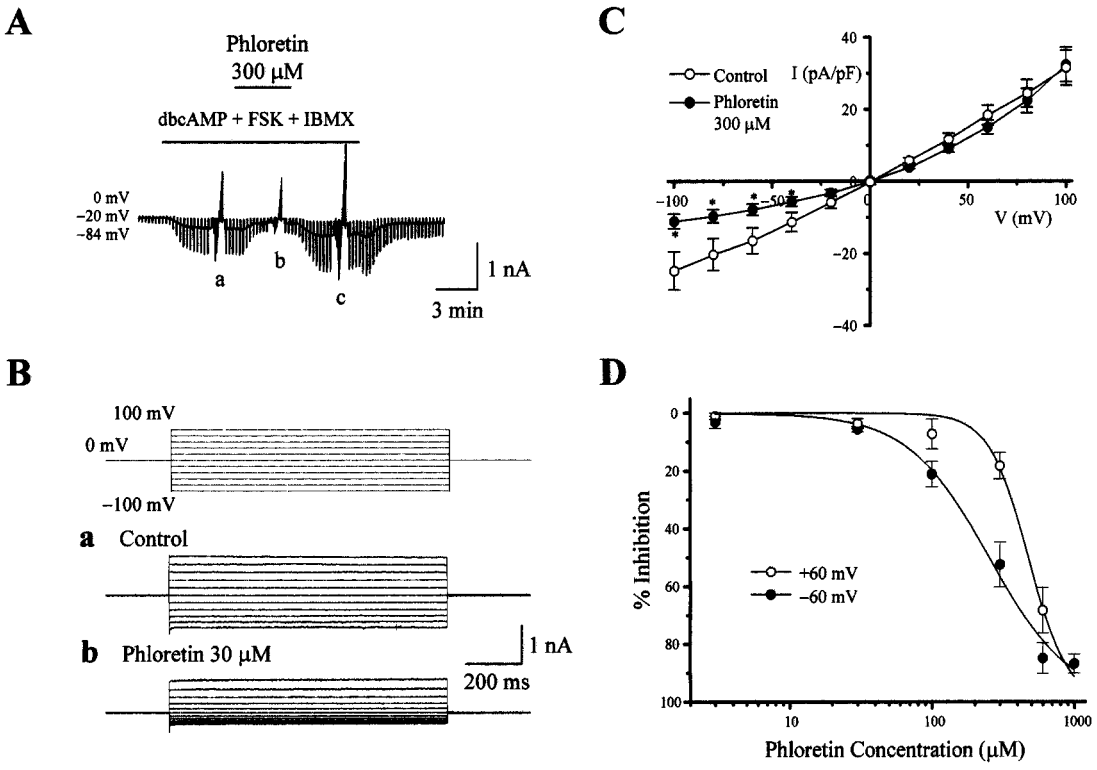


**Figure 7** Effects of extracellular 300 $\mu$M phloretin on cyclic AMP-activated whole-cell $Cl^-$ currents in T84 cells. (A) Typical record before and after application of a cocktail of forskolin (10 $\mu$M), dbcAMP (500 $\mu$M) and IBMX (400 $\mu$M) in the absence or presence of 300 $\mu$M phloretin (indicated by horizontal lines) in bath solution during application of alternating pulses between 0 mV and −84 mV from a holding potential of −20 mV or of step pulses (1-s duration) from −100 mV to +100 mV in 20-mV increments (at a, b & c). (B) Expanded traces of current responses (a & b in A) to step pulses in the absence (a) or presence of 300 $\mu$M phloretin (b). Inset: the step pulse protocol. (C) Current-voltage curves measured in the absence (control) or presence of 300 $\mu$M phloretin. Each symbol represents the mean current (with the s.e.mean: vertical bars) of eight experiments. *Significantly different from control experiments. (D) Concentration-inhibition curves for phloretin-induced inhibition of cyclic AMP-activated $Cl^-$ currents at −60 mV and +60 mV. Each symbol represents the mean ± s.e.mean ($n$ = 7–13). Data were fitted to a sigmoidal relation with the $IC_{50}$ values given in the text and with $h$ of 3.2 at +60 mV and 1.5 at −60 mV.

channels (Gribkoff *et al*., 1996). Activation of $Ca^{2+}$-dependent $K^+$ channels is known to be one of the causal factors leading to RVD in many epithelial cells (Okada & Hazama, 1989; Okada, 1997), as originally demonstrated in Intestine 407 cells (Hazama & Okada, 1988). Our recent study (Fan *et al*., 2000) using a high-speed automatic image analysing technique (Morishima *et al*., 1998), however, showed that phloretin (300 $\mu$M) completely inhibited the RVD of Intestine 407 cells under hypotonic conditions. Thus, there is a possibility that phloretin inhibits the RVD by impairing some other volume-regulatory ionic pathway. The present study demonstrated that phloretin, but not a glucoside of phloretin (phloridzin), can inhibit volume-sensitive $Cl^-$ channels at concentrations over 10 $\mu$M.

### *Phloretin does not inhibit $Ca^{2+}$-activated $Cl^-$ channels*

At 30–300 $\mu$M, phloretin never inhibited $Ca^{2+}$-activated $Cl^-$ currents in T84 cells (Figure 6). These results are in good agreement with previous observations with 50 $\mu$M phloretin in T84 cells (Worrell & Frizzell, 1991). Ionomycin-induced activation of $Ca^{2+}$-dependent $Cl^-$ currents persisted only for a short time and subsequently decayed rapidly despite persistent stimulation with ionomycin. A similar decay of $Ca^{2+}$-activated $Cl^-$ currents was observed before the cytosolic $Ca^{2+}$ decline in equine tracheal smooth muscle cells and reported to be due to phosphorylation mediated by $Ca^{2+}$/calmodulin-dependent kinase II (Wang & Kotlikoff, 1997). The present study showed that $Ca^{2+}$-activated $Cl^-$ currents became reactivated when phloretin was applied after inactivation or rundown of the current (Figure 6). The exact mechanism of phloretin-induced reactivation of the $Ca^{2+}$-activated $Cl^-$ current remains unknown. However, it is noted that phloretin enhanced the ionomycin-induced rise of intracellular $Ca^{2+}$ level, although phloretin alone never affected the cytosolic $Ca^{2+}$ concentration in T84 cells (K. Dezaki, H.-T. Fan & Y. Okada, 2001, unpublished observations).

### *Phloretin suppresses voltage-dependently cAMP-activated $Cl^-$ channels at high concentrations*

Phloretin preferentially suppressed the inward components of cyclic AMP-activated $Cl^-$ currents at high concentrations (over 100 $\mu$M) in both T84 (Figure 7) and C127/CFTR cells with $IC_{50}$ of 195 to 252 $\mu$M at −60 mV and of 355 to 475 $\mu$M at +60 mV. These results are in contrast to the previous observations of lack of detectable effect of 350 $\mu$M phloretin on CFTR $Cl^-$ currents expressed in *Xenopus* oocytes (Schreiber *et al*., 1997). The activity of CFTR $Cl^-$ channel is known to be dependent on phosphorylation by PKC

(Tabcharani *et al*., 1991; Winpenny *et al*., 1995; Jia *et al*., 1997). Although phloretin was reported to inhibit PKC (Gschwendt *et al*., 1984), an involvement of PKC in phloretin-induced suppression of cyclic AMP-activated $Cl^-$ currents is unlikely because: (1) the phloretin effect was voltage-dependent (Figure 7), (2) phloretin was ineffective when applied from the intracellular side, and (3) a PKC inhibitor, calphostin C, failed to inhibit cyclic AMP-activated $Cl^-$ currents.

### *Phloretin inhibits voltage-independently volume-sensitive $Cl^-$ channels at low concentrations*

At much lower concentrations (with $IC_{50}$ of around 30 $\mu$M), phloretin inhibited volume-sensitive $Cl^-$ currents in three different cell types in a voltage-independent manner. This effect should also be independent of PKC for the following reasons: (1) Phloretin failed to exert an inhibitory action when added to the intracellular solution in T84, Intestine 407 and C127/CFTR cells. (2) Volume-sensitive $Cl^-$ currents were never affected by phloridzin, which is known to act as a PKC inhibitor (Shoji *et al*., 1997), in Intestine 407 cells (Figure 5) and T84 cells. (3) In Intestine 407 cells, the activity of volume-sensitive $Cl^-$ channel was found to be insensitive to non-specific PKC blockers, polymixin B (Kubo & Okada, 1992) and H-7 (Okada *et al*., 1994). (4) A PKC-specific inhibitor, calphostin C, also never exhibited any inhibitory effect on volume-sensitive $Cl^-$ currents in Intestine 407 cells.

Part of the chemical structure of the phloretin molecule resembles a stilbene-derivative $Cl^-$ channel blocker (DIDS or SITS), especially in the two benzene rings (see Figure 8). However, the blocking mechanism of phloretin was distinct from that of DIDS or SITS which shows voltage-dependent (open-channel block-like) inhibition for volume-sensitive $Cl^-$ channels in many cell types (Okada, 1997). Since phloridzin was totally ineffective, the hydroxyl residue, at which D-glucose binds to a benzene ring of phloridzin, might play an essential role in the phloretin-induced blocking action.

Although phloretin was also shown to inhibit partially cyclic AMP-activated $Cl^-$ currents (preferentially the inward component), much higher concentrations (over 100 $\mu$M: Figure 6D) were required. In contrast, phloretin is known to block anion exchanger ($Cl^-/HCO_3^-$ antiporter: AE) at micromolar concentrations (Fröhlich & Gunn, 1987). Thus, at concentrations lower than 100 $\mu$M, phloretin may serve as a blocker of anion transport not only *via* electroneutral AE but also *via* volume-sensitive $Cl^-$ channels, and thus as a useful tool to discriminate the contribution of volume-sensitive $Cl^-$ channels in the electroconductive anion transport from that of $Ca^{2+}$ - or cyclic AMP-activated $Cl^-$ channels. However, it must be noted that phloretin can also suppress several types of voltage-gated cation channels (Strichartz *et al*., 1980; Klusemann & Meves, 1991; 1992; Prevarskaya *et al*., 1994). Thus, it is expected that a more selective derivative of phloretin, if it were discovered, might offer a promising tool for purification of the volume-sensitive $Cl^-$ channel protein, the gene of which has not been precisely identified as yet (Okada, 1997; Okada *et al*., 1998).

Phloretin was shown to inhibit the RVD of Intestine 407 cells (Fan *et al*., 2000). In this cell line, the RVD is known to be attained by operation of the following ion channels in concert: volume-sensitive $Cl^-$ channels (Kubo & Okada, 1992), $Ca^{2+}$-activated $K^+$ channels (Hazama & Okada, 1988), and $Ca^{2+}$-permeable non-selective cation channels (Okada *et al*., 1990). However, phloretin has been reported to enhance, but not suppress, $Ca^{2+}$-activated $K^+$ channels in other cell types (Koh *et al*., 1994; Gribkoff *et al*., 1996; Shin *et al*., 1996; Bringmann & Reichenbach, 1997). Since a swelling-induced increase in cytosolic $Ca^{2+}$, which is known to be triggered by $Ca^{2+}$ influx through non-selective cation channels in Intestine 407 cells (Okada *et al*., 1990), was still observed in the presence of phloretin in our preliminary fura-2 study in T84 cells (K. Dezaki, H.-T. Fan & Y. Okada, 2001, unpublished observations), it is unlikely that phloretin inhibits swelling-induced activation of $Ca^{2+}$-permeable non-selective cation channels. Thus, inhibition of volume-sensitive $Cl^-$ channel by phloretin may mainly explain how this chemical inhibits RVD.

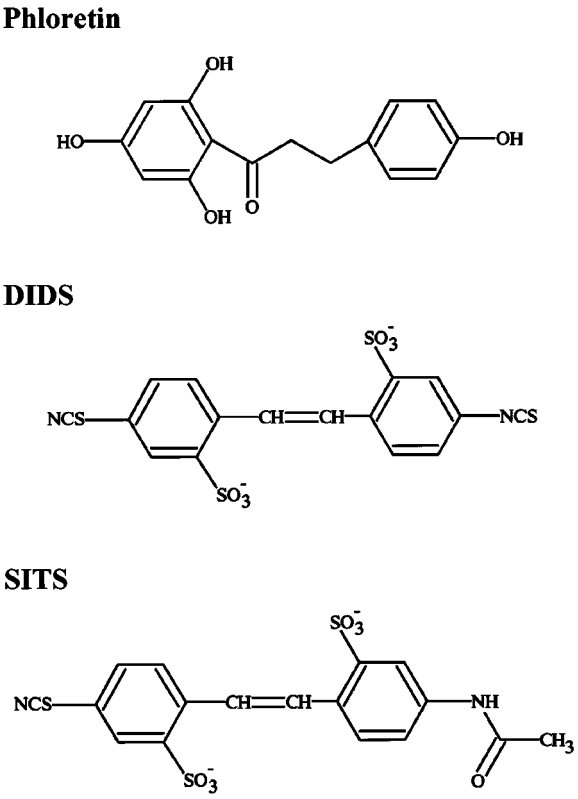


**Figure 8** Structures of phloretin, SITS and DIDS.

### *Conclusion*

Phloretin, which is a well-known inhibitor of glucose uniporter and anion aniporter and known to affect voltage-gated cation channels, inhibits volume-sensitive $Cl^-$ channels in a voltage-independent manner at low concentrations (over 10 $\mu$M) in epithelial cells. In addition, the drug partially inhibited cyclic AMP-activated CFTR $Cl^-$ channels in a voltage-dependent manner at higher concentrations (over 100 $\mu$M). In contrast, phloretin never inhibited $Ca^{2+}$-activated $Cl^-$ channels. It is concluded that phloretin is a potent $Cl^-$ channel blocker in epithelial cells, preferentially blocking the volume-sensitive $Cl^-$ channel at concentrations below 100 $\mu$M.

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