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Roles of Volume-sensitive Cl^- Channel in Cisplatin-induced Apoptosis in Human Epidermoid Cancer Cells

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Abstract. The anti-cancer drug cisplatin induces apoptosis by damaging DNA. Since a stilbene-derivative blocker of Cl^-/HCO_3^- exchangers and Cl^- channels, SITS, is known to induce cisplatin resistance in a manner independent of intracellular pH and extracellular HCO_3^-, we investigated the relation between cisplatin-induced apoptosis and Cl^- channel activity in human adenocarcinoma KB cells. A stilbene derivative, DIDS, reduced cisplatin-induced caspase-3 activation and cell death, which were detected over 18 h after treatment with cisplatin. DIDS was also found to reduce sensitivity of KB cells to 5-day exposure to cisplatin. Whole-cell patch-clamp recordings showed that KB cells functionally express volume-sensitive outwardly rectifying (VSOR) Cl^- channels which are activated by osmotic cell swelling and sensitive to DIDS. Pretreatment of the cells with cisplatin for 12 h augmented the magnitude of VSOR Cl^- current. Thus, it is concluded that cisplatin-induced cytotoxicity in KB cells is associated with augmented activity of a DIDS-sensitive VSOR Cl^- channel and that blockade of this channel is, at least in part, responsible for cisplatin resistance induced by a stilbene derivative.

Key words: Cl^- channel — Apoptosis — Cisplatin — Cancer cell

Introduction

cis-Diaminedichloroplatinum (cisplatin) is a platinum-based DNA-damaging agent that is widely used in chemotherapy for a variety of solid tumors (Loehrer & Einhorn, 1984) and is thought to exert its cytotoxic action by crosslinking to DNA and forming intra- and inter-strand adducts (Chu, 1994; Jamieson & Lippard, 1999). Like most chemotherapeutic agents, the anticancer activity of cisplatin is known to be attained by initiating apoptosis (Barry, Behnke & Eastman, 1990; Eastman, 1990; Sorenson, Barry & Eastman, 1990; Sklar & Prochownik, 1991). The exact mechanisms by which cisplatin induces apoptosis are not completely understood.

4-Acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), a stilbene derivative that is a known blocker of both Cl^-/HCO_3^- exchangers and Cl^- channels, was reported to induce resistance to cisplatin in mouse mammary tumor cells (Laurencot & Kennedy, 1995) and canine osteosarcoma cells (Yarbrough et al., 1999). Since this effect of SITS was independent of intracellular pH (Laurencot & Kennedy, 1995) and extracellular HCO_3^- (Yarbrough et al., 1999), it seems likely that some Cl^- channel is the target of SITS. Recently, we have demonstrated that both mitochondrion- and death receptor-mediated apoptosis is triggered by activation of the volume-sensitive outwardly rectifying (VSOR) Cl^- channel (Shimizu, Numata & Okada, 2004), which is sensitive to the stilbene derivatives SITS and 4-4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) (Okada, 1997). Thus, there arises the possibility that cisplatin-induced cytotoxicity involves the activity of the VSOR Cl^- channel and that stilbene derivative-induced cisplatin resistance is mediated by blockade of the VSOR Cl^- channel. In the present study, this possibility was tested using human epidermoid adenocarcinoma KB cells.

Materials and Methods

Cell Culture

Human epidermoid cancer KB cells were cultured in monolayers in plastic flasks in Eagle's minimal essential medium (Nissui, Tokyo,

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Japan) containing 10% fetal bovine serum, as described previously (Fujii et al., 1994). For patch-clamp studies, suspensions of spherical cells were prepared by mechanical detachment from the plastic flasks, as described previously (Kubo & Okada, 1992), and after suspension culture with agitation for 15–240 min the cells were placed in a glass-bottomed chamber (0.3 ml volume).

Cell Viability Assay

To examine the time-dependent change of cell viability after treatment with cisplatin, KB cells were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated in the absence of any drugs for 24 h. The cells were then exposed to medium containing cisplatin (15 μM), with or without DIDS (100 μM). Every 6 h until 24 h, cell viability was assessed by the colorimetric MTT assay using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

To test their sensitivity to cisplatin, KB cells were seeded in 96-well plates at a density of 5×10^2 cells/well and incubated in the absence of any drugs for 24 h. The cells were then exposed to medium with various concentrations of cisplatin in the absence or presence of DIDS (100 μM) for 5 days. Viability of cells was assessed using the same kit.

Caspase-3 Activity Measurements

Caspase-3 activity was measured by using a fluorometric assay (Thornberry, 1994). To exclude an involvement of other related proteases, the difference in fluorescence from samples with a specific inhibitor of caspase-3 absent and present was observed. The fluorogenic substrate for caspase-3 (Ac-DEVD-AMC), which was labeled with the fluorochrome 7-amino 4-methyl coumarin (AMC), and the tetrapeptide inhibitor of caspase-3 (Ac-DEVD-CHO) were provided in the CaspACE Assay System (Promega, Madison, WI). To examine the time-dependent effects of cisplatin and the effects of Cl^- channel blockers, KB cells were exposed to cisplatin for 24 h in the presence or absence of DIDS (100 μM) or phloretin (100 μM).

Whole-Cell Cl^- Current Measurements

Volume-sensitive Cl^- currents were recorded at room temperature (22–26°C) by the whole-cell patch-clamp technique, as previously described (Kubo & Okada, 1992; Shimizu, Morishima & Okada, 2000). The patch electrodes were fabricated from borosilicate glass capillaries using a micropipette puller (P-2000, Sutter Instruments, Novato, CA) and had a resistance of around 2 MΩ, when filled with pipette solution. pCLAMP software (Axon Instruments, Union City, CA) was used for command pulse control, data acquisition and analysis. The time course of current activation and recovery was monitored by repetitively applying alternating pulses of ±40 mV (6 s duration) from a holding potential of 0 mV. To monitor the voltage dependence of the current, step pulses (2 s duration) were sometimes applied from a pre-potential of −100 mV (0.5 s duration) to test potentials of −100 to +100 mV in 20-mV increments. To determine the reversal potentials, ramp command voltages from −50 to +50 mV were applied after steady current activation was observed in association with steady osmotic swelling. Currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), filtered at 1 kHz and digitized at 4 kHz. Series resistance (< 10 MΩ) was compensated (to 60–70%) to minimize voltage errors. To eliminate K^+ currents, Cs-rich solutions were employed. Pipette (intracellular) solution consisted of (in mM) 110 CsCl, 1 Na_2ATP, 15 Na-HEPES, 10 HEPES, 2 $MgSO_4$, 1 EGTA and 50 mannitol (300 mosmol $(kg\text{-}H_2O)^{-1}$, pH 7.3). Isotonic bathing solution contained (in mM) 110 CsCl, 12 HEPES, 7 Tris, 5 $MgSO_4$, and 100 mannitol (330 mosmol $(kg\text{-}H_2O)^{-1}$, pH 7.5). To prevent spontaneous cell swelling after attaining the whole-cell mode, presumably due to poorly diffusible cytosolic macromolecules (Worrell et al., 1989), the osmolality of the pipette solution was set lower than that of the isotonic bathing solution. Cell swelling was induced by hypotonic (270 mosmol $(kg\text{-}H_2O)^{-1}$) bathing solution in which mannitol was reduced to 40 mM. In some experiments, CsCl was replaced with NMDG-Cl, CsCl or CsBr in the hypotonic bathing solution.

Statistical Analysis

Data are given as means ± SEM of n observations. Statistical differences of the data were evaluated by Student's paired or unpaired t-test and considered significant at $P < 0.05$.

Results

Cisplatin-induced Apoptosis and Its DIDS Sensitivity

When evaluated by activation of caspase-3, apoptosis did not occur in KB cells up to 12 h of treatment with 15 μM cisplatin and started at ≥18 h of treatment, as shown in Fig. 1*A*. Cisplatin-induced cell death was also detected at ≥18 h of cisplatin treatment (Fig. 1*B*).

When a stilbene derivative, DIDS (100 μM), was simultaneously administered, cisplatin-induced apoptosis was prominently inhibited. Caspase-3 activation induced by 18- or 24-h exposure to cisplatin was markedly inhibited by DIDS, as shown in Fig. 1*A*. Cisplatin-induced activation of caspase-3 was also significantly suppressed by phloretin (100 μM), which is known to be more selective for VSOR Cl^- channels than for cAMP- or Ca^{2+}-activated Cl^- channels (Fan et al., 2001). Cell death induced by cisplatin was largely prevented by DIDS (Fig. 1*B*).

Cisplatin Sensitivity and Its DIDS Sensitivity

When KB cells were exposed to cisplatin for 5 days, apoptotic cell death was induced by cisplatin at much lower concentrations, as shown in Fig. 2. The half-maximal effective concentration (EC_{50}) was 0.49 ± 0.01 μM ($n = 3$), which is comparable to the dose required to reduce the initial surviving fraction to 10% as examined by a colony formation assay after 10 days (0.38 μM: Matsuo et al., 1990).

In the presence of 100 μM DIDS, KB cells became more resistant to 5-day exposure to cisplatin (EC_{50} = 0.84 ± 0.01 μM: Fig. 2).

Cisplatin-induced Augmentation of Swelling-Activated, DIDS-Sensitive Cl^- Currents

Under isotonic conditions, in which the osmolalities of the bathing and pipette solutions were kept at 330 and 300 mosmol $(kg\text{-}H_2O)^{-1}$, respectively, no appreciable current was observed in KB cells in the whole-

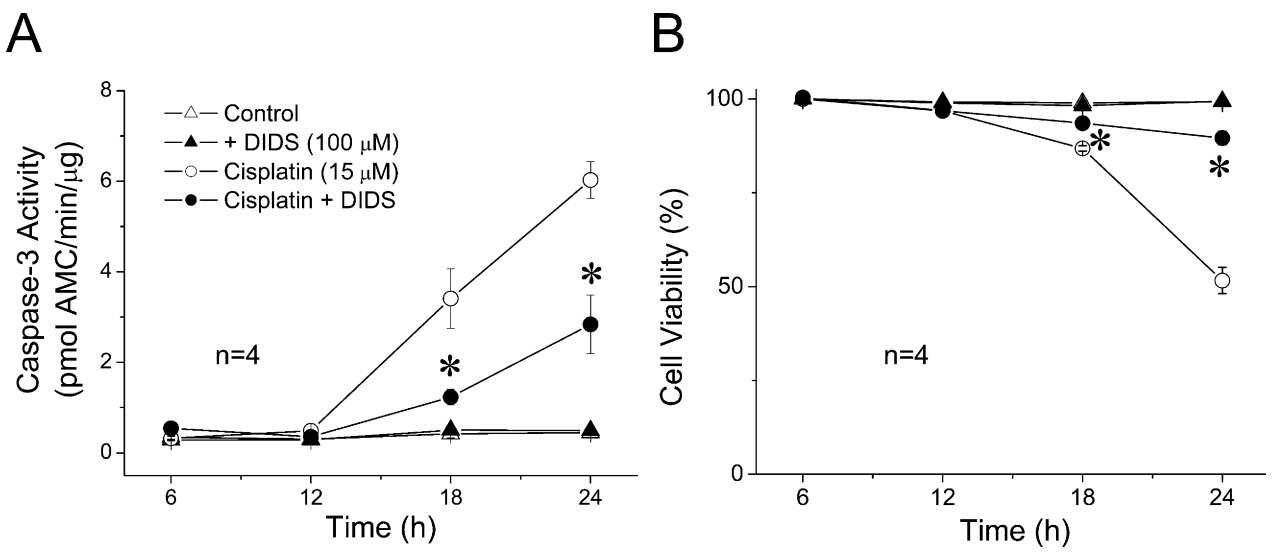

Fig. 1. Effects of DIDS on cisplatin-induced activation of caspase-3 activity (*A*) and reduction of cell viability (*B*) in KB cells. Each symbol represents the mean ± SEM (vertical bar). *, significant difference ($P < 0.02$) between data obtained in the absence and presence of 100 μM DIDS, in cisplatin-treated cells.

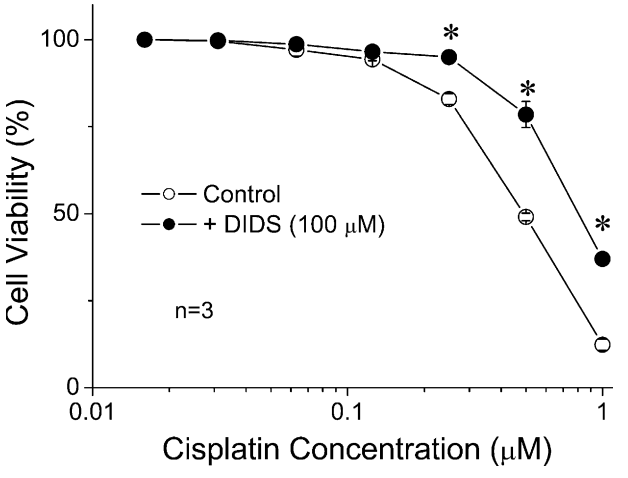

Fig. 2. Effects of DIDS on sensitivity of KB cells to 5-day exposure to cisplatin. Each symbol represents the mean ± SEM (vertical bar). *, significantly different ($P < 0.005$) from the control data. EC_{50} values are 0.49 ± 0.01 μM and 0.84 ± 0.01 μM for data in the absence and presence of 100 μM DIDS, respectively.

cell configuration. However, when osmotic swelling was induced by applying a hypotonic bathing solution of 270 mosmol $(kg\text{-}H_2O)^{-1}$, whole-cell currents gradually increased in a reversible manner (Fig. 3*A*, top panel). In response to voltage-step pulses, currents were elicited that exhibited moderate outward rectification and time-dependent inactivation at positive potentials larger than +60 mV (Fig. 3*A*, bottom panel). The *I-V* curve obtained by ramp clamp also exhibited outward rectification with a reversal potential of around 0 mV (Fig. 3*B*, Cl^-). Virtually no change was observed in the *I-V* curve by replacing extracellular Cs^+ with a large cation, $NMDG^+$ (*data not shown*), indicating the currents were anionic. In contrast, substitution of extracellular Cl^- with I^- or Br^- reduced the slope conductance of the *I-V* curve and shifted the reversal potential to more negative values (Fig. 3*B*). The anion permeability coefficients estimated from the reversal potentials were $I^- : Br^- : Cl^- = 1.37 \pm 0.08 : 1.10 \pm 0.02 : 1$. These biophysical properties are identical to those of the volume-sensitive outwardly rectifying (VSOR) anion channel (Okada, 1997).

The pharmacological properties of currents recorded in swollen KB cells were also identical to those of the VSOR Cl^- channel. As shown in Fig. 4*A*, a stilbene derivative, DIDS (100 μM), blocked the swelling-activated current (left panel) in a voltage-dependent manner (right panel). The outward current was more sensitive to DIDS than the inward current, and inactivation kinetics of the outward current recorded at a large positive potential were accelerated by DIDS (*data not shown*). In contrast, as shown in Fig. 4*B*, the swelling-activated currents were blocked by 100 μM phloretin in a voltage-independent manner.

In KB cells pretreated with 15 μM cisplatin for 12 h, similar VSOR Cl^- currents were recorded under hypotonic conditions after washout of cisplatin. Swelling-activated currents were inhibited by DIDS and phloretin in voltage-dependent and –independent manner, respectively (Fig. 5).

As shown in Fig. 6*A*, the current activation induced by cell swelling was reversible (top panel) and exhibited outward rectification and inactivation kinetics at large positive potentials (bottom panel). The anion permeability sequence of I^- (1.31 ± 0.03) > Br^- (1.06 ± 0.01) > Cl^- (1) estimated from the

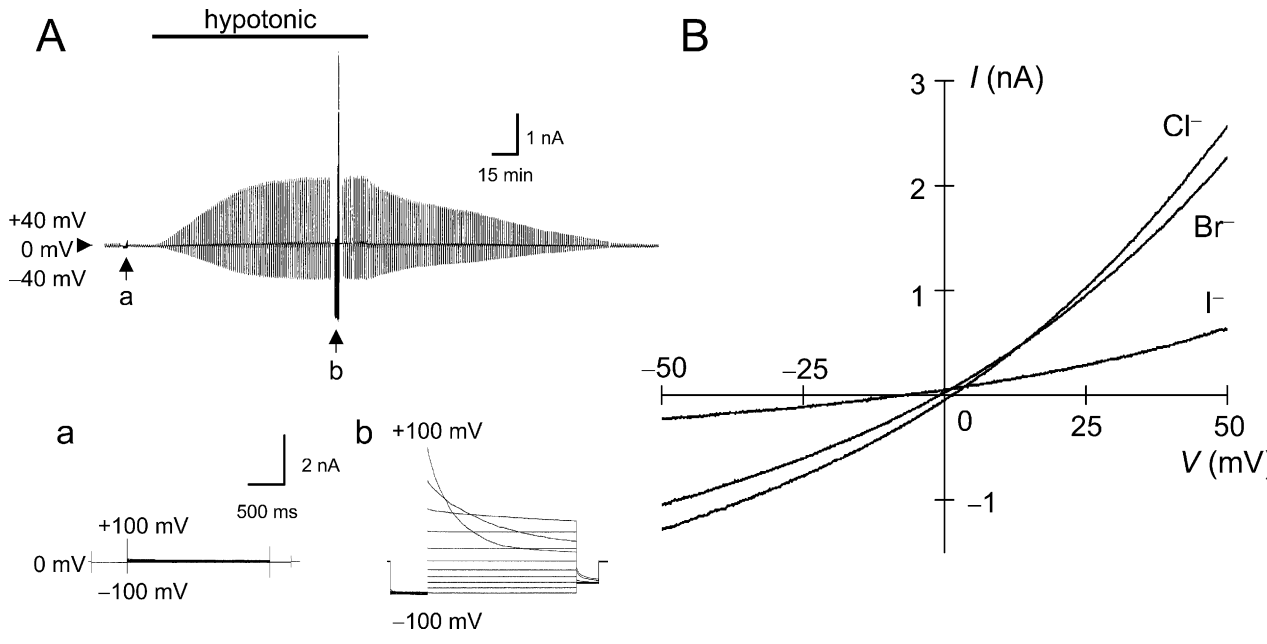

Fig. 3. Swelling-activated VSOR Cl^- currents in KB cells. (*A*) *Upper panel:* representative record of currents before, during and after a hypotonic challenge (90% osmolality). Alternating pulses from 0 to ±40 mV or step pulses (at *arrows*) from −100 to +100 mV in 20-mV increments were applied. *Lower panel:* expanded traces of current responses to step pulses (at *a* and *b*). (*B*) Effects of anion substitutions on current-voltage (*I-V*) curves measured by ramp voltage clamp.

reversal potentials in cisplatin-pretreated cells (Fig. 6*B*) was also similar to that for KB cells not pretreated with cisplatin (Fig. 3*B*). As summarized in Fig. 6*C*, however, the current density recorded in cisplatin-pretreated KB cells (*filled circles*) was profoundly larger than that in control KB cells (*empty circles*).

Discussion

A stilbene derivative, SITS, was previously shown to induce cisplatin resistance in tumor cells in a manner independent of intracellular pH (Laurencot & Kennedy, 1995) and of ambient HCO_3^- (Yarbrough et a1., 1999). Since stilbene derivatives block not only Cl^-/HCO_3^- exchangers but also Cl^- channels, we hypothesized that stilbene derivative-induced cisplatin resistance is mediated by blockade of some Cl^- channel. In the present study, in fact, another stilbene derivative, DIDS, was found to suppress cisplatin-induced apoptosis in human epidermoid cancer KB cells (Fig. 1). Also, a non-stilbene-derivative blocker of the VSOR Cl^- channel, phloretin, was observed to inhibit cisplatin-induced activation of caspase-3 in KB cells. Furthermore, DIDS was found to reduce sensitivity of KB cells to 5-day exposure to cisplatin (Fig. 2). KB cells were found to express the VSOR Cl^- channel (Fig. 3), which exhibited sensitivity to DIDS and phloretin (Fig. 4). Furthermore, the activity of the VSOR Cl^- channel was prominently augmented by pretreatment (for 12 h) with cisplatin (Figs. 5 & 6). It thus appears that cisplatin-induced cytotoxicity and stilbene derivative-induced cisplatin resistance are associated with augmentation and suppression, respectively, of the VSOR Cl^- channel, which is known to be involved in the induction of apoptosis (Shimizu et al., 2004).

Cisplatin is known to remain in its electroneutral chloride-bound forms, which are not reactive with the cell, in the extracellular solution, which contains a high concentration of Cl^-; but once it has entered the cytosol, which has a lower Cl^- concentration, it is hydrolyzed to its charged aqua forms, which attack polar donor groups in the DNA target molecule (Miller & House, 1990). Thus, there exists a possibility that the effect of DIDS may, at least in part, be caused by an increase in the intracellular Cl^- concentration. However, this possibility is unlikely, because blockade of a Cl^- channel per se cannot induce elevation of the cytosol Cl^- level.

The apoptotic volume decrease (AVD), which precedes caspase activation, DNA laddering and cell fragmentation into apoptotic bodies, is known to be a prerequisite to apoptosis and to be induced by activation of volume-regulatory K^+ and Cl^- channels (Maeno et al., 2000; Okada et al., 2001). The AVD-inducing Cl^- channel was functionally identified as the VSOR Cl^- channel for both mitochondrion-mediated and death receptor-mediated apoptosis in human epithelial HeLa cells (Shimizu et al., 2004). In the present study, VSOR Cl^- channel activity was found to be associated with apoptosis

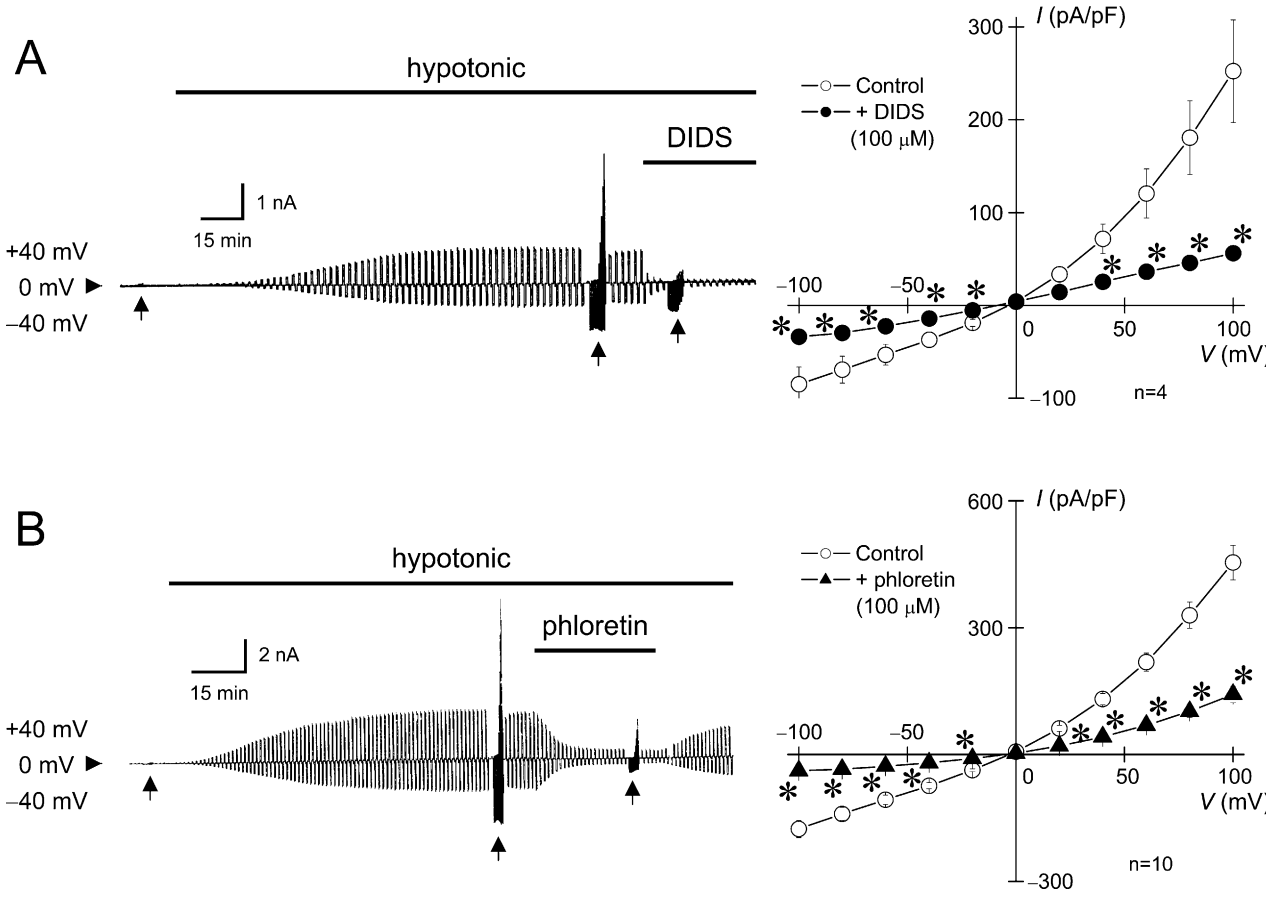

Fig. 4. Effects of DIDS (*A*) and phloretin (*B*) on swelling-activated VSOR Cl^- currents in KB cells. *Left panels:* representative records of currents before, during and after a hypotonic challenge in the absence and presence of 100 μM DIDS or 100 μM phloretin. Pulses were applied as in Fig. 3*A*. Right panels: *I-V* relationships for instantaneous currents in the absence and presence of DIDS or phloretin. Each symbol represents the mean ± SEM (vertical bar). *, significantly different ($P < 0.05$) from the control data.

induced by an inducer of DNA damage-mediated apoptosis, cisplatin, in human epidermal KB cells.

It is unclear how cisplatin induces activation of VSOR Cl^- channels. Cisplatin was reported to activate a variety of cellular signalling molecules including c-Abl tyrosine kinase (Kharbanda et al., 1998), Lyn tyrosine kinase (Yoshida et al., 2000), the tumor suppressor p53 protein (Kharbanda et al., 1998; Sanchez-Prieto et al., 2000; Cummings & Schnellmann, 2002), p38 MAP kinase (Pandey et al., 1996; Sanchez-Prieto et al., 2000; Deschesnes et al., 2001), MEKK1 (Kharbanda et al., 2000), SEK1 (Sanchez-Perez & Perona, 1999), JNK/SAPK (Kharbanda et al., 1995; Pandey et al., 1996; Sanchez-Perez et al., 2000), ERK (Persons et al., 1999; Wang, Martindale & Holbrook, 2000), and reactive oxygen species (Ravi, Somani & Rybak, 1995; Tsutsumishita et al., 1998; Tsuruya et al., 2003). Some of these molecules might regulate the activity of the VSOR Cl^- channel. Since the VSOR Cl^- channel and components of its activation mechanism may provide promising targets for the development of anticancer drugs, identification of these molecules in future studies is of great importance.

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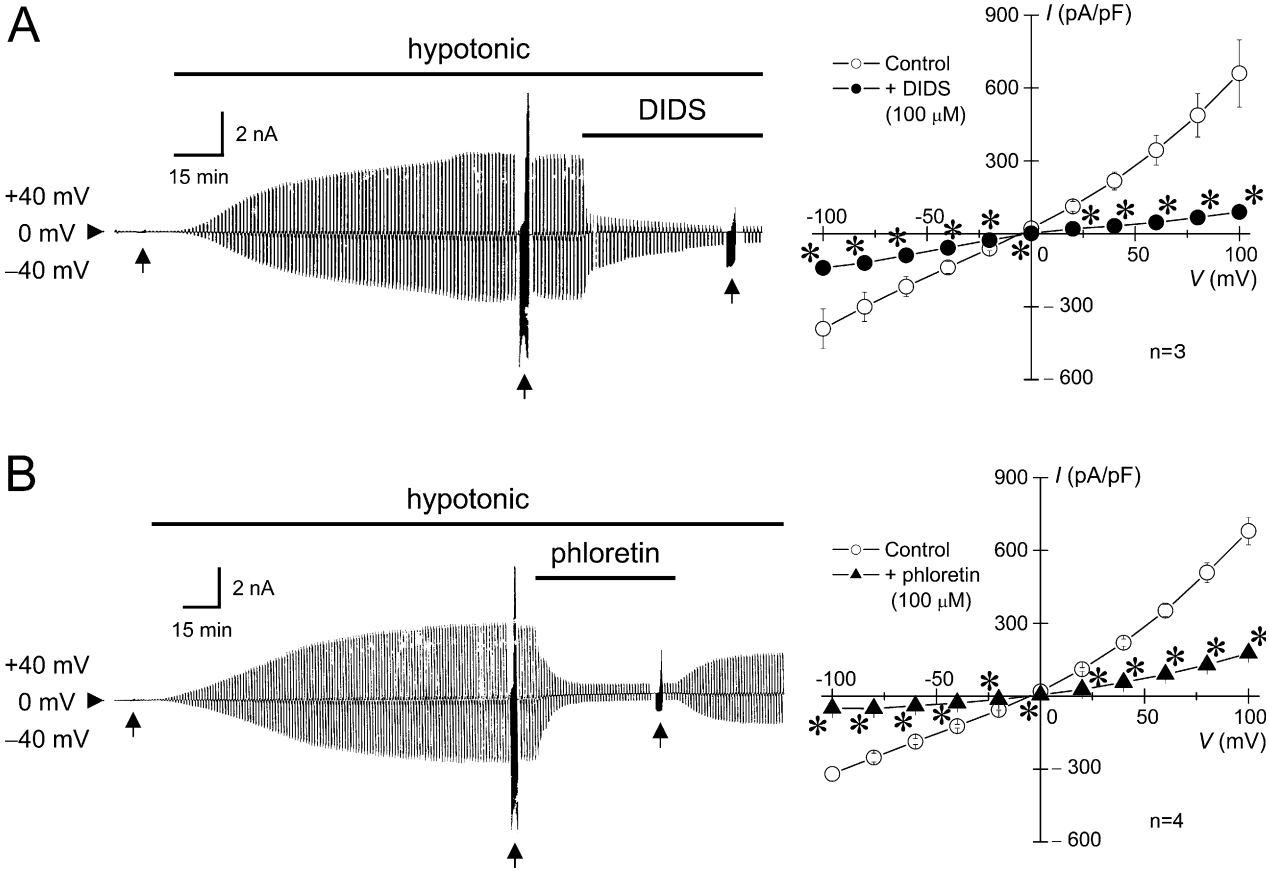

Fig. 5. Effects of DIDS (*A*) and phloretin (*B*) on swelling-activated VSOR Cl^- currents in KB cells pretreated with cisplatin (15 μM for 12 h). Explanation of figure is otherwise the same as in Fig. 4.

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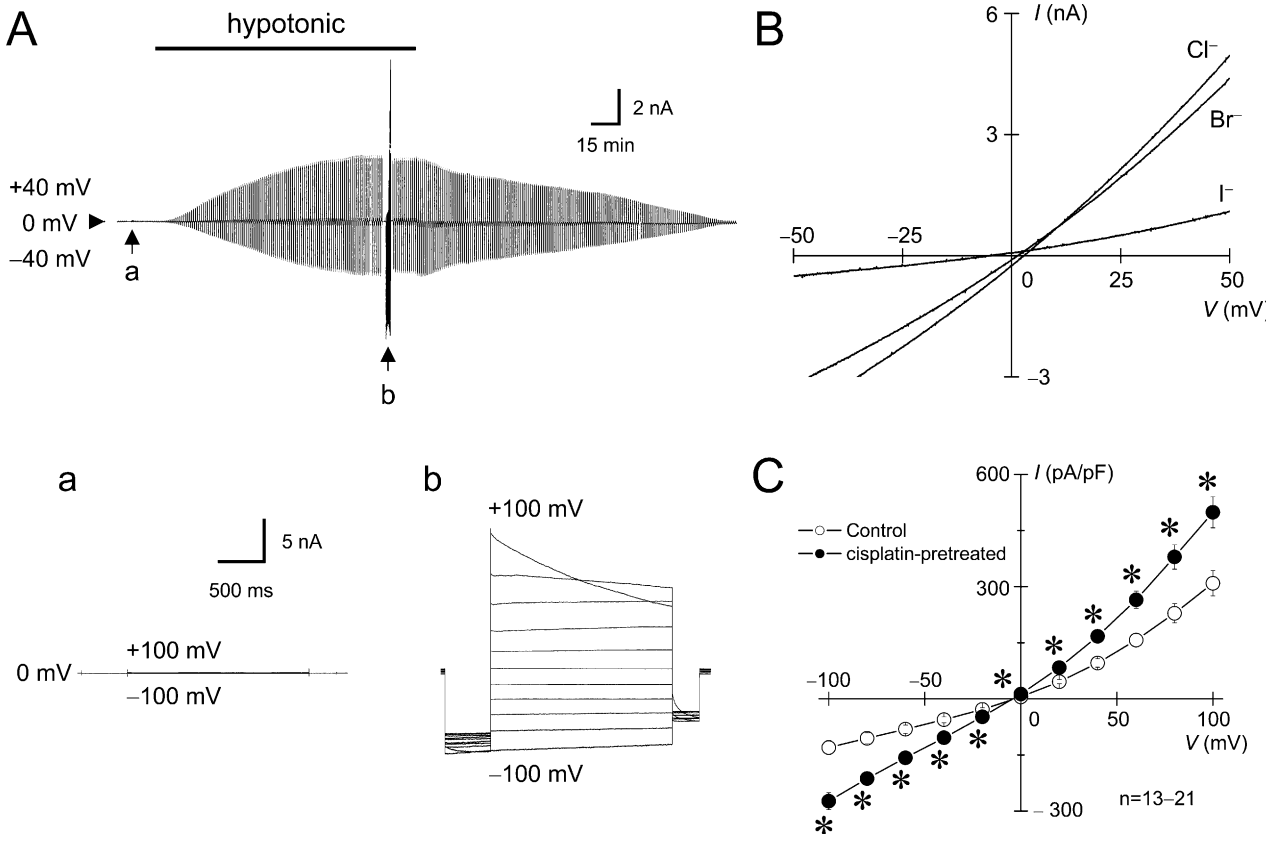

Fig. 6. Swelling-activated VSOR Cl^- currents in KB cells pretreated with cisplatin. (*A*) and (*B*), same as in Fig. 3. (*C*) *I-V* relationships for instantaneous currents activated in response to step pulses applied to control KB cells (*empty circles*) and KB cells pretreated with cisplatin (*filled circles*). *, significantly different ($P < 0.05$) from the control data.

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