Alteration of gene expression in cisplatin-resistant KB cells and functional consequences:

Upregulation of the volume-sensitive outwardly rectifying chloride channel and enhancement of apoptosis by a histone deacetylase inhibitor

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Summary

Resistance of cancer cells to drug treatment is a major problem in cancer therapy. The platinum-based drug cisplatin (cis-diamminedichloroplatinum(II)) is a widely used anticancer drug introduced more than 20 years ago which acts mainly by forming adducts with DNA that cause the induction of apoptosis. However, some types of cancer have high intrinsic resistance to cisplatin; in addition, resistance to the drug frequently appears in initially responsive tumors. The mechanisms of resistance to cisplatin are the subject of intense study because their elucidation will lead to the development of better, more effective treatments for cancer.

A model of cisplatin resistance is provided by the cisplatin-resistant KB (KB/CP4) cell line, which is derived from human KB epidermoid cancer cells. It was found previously in our laboratory that activity of the volume-sensitive, outwardly rectifying (VSOR) chloride channel is virtually absent in these cells. Because VSOR chloride current activation is known to be essential to the progression of apoptosis, it was hypothesized that the absence of the current contributes to the cisplatin resistance of these cells.

An attempt was first made to determine the molecular identity of the VSOR chloride channel or a regulator of it. A strategy for identifying the molecule(s) was suggested by the fact that the KB/CP4 cell line, unlike the KB cell line from which it is derived, does not express volume-sensitive chloride current. Using a subtractive hybridization method, candidates for differentially expressed genes were identified. With semi-quantitative RT-PCR, seven genes were confirmed as being expressed at a higher level in KB cells than in KB/CP4 cells; five of these were genes which had been identified by subtractive hybridization. The genes confirmed as having higher expression in KB cells were lactate dehydrogenase A (LDHA), S100 calcium binding protein A4 (S100A4), thymosin β4 (TMSB4X), argininosuccinate synthetase (ASS), placental alkaline phosphatase (ALPP),

folate receptor 1 (FOLR1) and GPI-specific phospholipase D1 (GPLD1). All of these genes, except for ALPP, were then cloned into expression vectors and overexpressed in KB/CP4 cells to determine if they could restore VSOR chloride current expression. The overexpression of these genes did not result in restoration of the current in KB/CP4 cells, however.

Because VSOR chloride current could not be restored in KB/CP4 cells by gene overexpression, treatment with drugs that remove gene silencing was tested in an attempt to restore the current. Epigenetic changes which alter chromatin structure--gene promoter methylation and histone deacetylation, in particular--are known to cause inhibition of gene expression, and could potentially contribute to the silencing of genes in KB/CP4 cells. 5-Aza-2'-deoxycytidine (Aza-dC) is a drug that reverses DNA methylation by inhibiting DNA methyltransferases, and trichostatin A (TSA) is a drug that reverses histone deacetylation by inhibiting histone deacetylases (HDACs). In KB/CP4 cells treated with Aza-dC, a volume-sensitive chloride current was not induced; however, it was found that the current could be induced in cells treated with TSA. This current was confirmed as VSOR chloride current based on its volume sensitivity, intracellular ATP dependency and sensitivity to two blockers, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and phloretin. It was found that in addition to this phenotypic change in the expression of VSOR chloride current, alterations in the expression of some genes occurred due to TSA treatment. As assessed by semi-quantitative RT-PCR, two genes previously identified as differentially expressed in KB and KB/CP4 cells, FOLR1 and GPLD1, were upregulated in TSA-treated KB/CP4 cells.

The effects of restoration of VSOR chloride current on cisplatin resistance in these cells were checked by a cell viability assay and caspase-3 activity assay. The viability of cells treated for 48 h with both TSA and cisplatin decreased to a greater extent than that of cells treated with TSA alone, and this decrease could be blocked by treatment with DIDS. Moreover, simultaneous treatment with

both TSA and cisplatin for 24 h was found to induce apoptosis in this cisplatin-resistant cell line: caspase-3 activity in cells treated for this length of time with both drugs was elevated compared to that in cells treated with TSA alone. This increase in caspase-3 activity could also be blocked by DIDS. These results demonstrate that cisplatin resistance in KB/CP4 cells treated with the HDAC inhibitor TSA decreases as a result of an increase in apoptosis, at least in part; in other words, TSA caused a sensitization of the cisplatin-resistant tumor cells to cisplatin-induced apoptosis. It is thus suggested that the activity of the VSOR chloride channel is essential for the TSA-induced increase in apoptosis.

In conclusion, this study provides evidence that the absence of VSOR chloride channel activity contributes to cisplatin resistance in a cisplatin-resistant KB cell line, and that restoration of VSOR chloride current in these cells is involved in sensitization to cisplatin-induced apoptosis in this tumor cell line. The identities of genes involved in the functioning of the channel remain to be clarified.

Introduction

Cisplatin (cis-diamminedichloroplatinum(II)) is a platinum-based drug widely used in the treatment of various types of cancer (Rozencweig et al., 1977; Loehrer and Einhorn, 1984). It is particularly effective in the treatment of testicular cancer, but is also used to treat ovarian, cervical, head and neck, and non-small-cell lung cancer, in addition to other types of cancer. It is thought to kill cancer cells mainly by forming adducts with DNA which interfere with DNA repair mechanisms (Roberts and Thomson, 1979; Chu, 1994; Jamieson and Lippard, 1999), leading to apoptosis (Barry et al., 1990; Eastman, 1990; Sorenson et al., 1990; Sklar and Prochownik, 1991; Mizushima et al., 1996; Van Waardenburg et al., 1996; Guchelaar et al., 1998).

While some types of cancer are sensitive to cisplatin, others are resistant. Cisplatin treatment is curative for testicular cancer in nearly all cases because of the sensitivity of this type of cancer to the drug (Chaganti and Houldsworth, 2000). Ovarian cancer, on the other hand, has high intrinsic resistance to treatment with cisplatin (Ozols and Young, 1984). In addition to such intrinsic resistance to cisplatin, acquired resistance to anticancer drugs is a major obstacle in the treatment of cancer (Loehrer and Einhorn, 1984; Ozols, 1992; Perez et al., 1993). In cases of drug resistance, current therapeutic strategies focus on testing different combinations of drugs, rather than on reversing resistance (Wernyj and Morin, 2004).

The mechanisms of resistance to cisplatin are complex, and are thought to involve inactivation of cisplatin, increased DNA repair, reduced intracellular accumulation of cisplatin, increased tolerance to cisplatin adducts and disruption of the apoptotic program (Timmer-Bosscha et al., 1992; Chao, 1996; Kartalou and Essigman, 2001; Wernyj and Morin, 2004; Wang and Lippard, 2005). Signalling pathways that are activated in response to DNA damage can make different contributions depending on the cell type (Dempke et al., 2000). For

example, the p53 signalling pathway, which has a crucial role in tumor suppression, appears to be important in cisplatin sensitivity in many cell types but not in others (Wang and Lippard, 2005). Impairment of this pathway may thus be a factor in cisplatin resistance in many but not all cases.

Previous data from our laboratory suggest that the effects of cisplatin are related to the functional expression of the volume-sensitive, outwardly rectifying (VSOR) chloride channel. It was found that VSOR chloride current is enhanced in human KB epidermoid tumor cells treated with cisplatin (Ise et al., 2002); in addition, the expression of VSOR chloride current is virtually absent in a cisplatin-resistant variant of these cells (KB/CP4 cells) which can survive and be cultured in the presence of cisplatin (Ise et al., 2002).

Activity of the VSOR chloride channel has been shown to be critical for cell volume regulation (Okada, 1997; Nilius et al., 1997; Strange et al., 1996). In response to a hypotonic stimulation which causes swelling, animal cells regulate their volume by a process called regulatory volume decrease (RVD) which occurs after the osmotic swelling. In RVD, the efflux of Cl^- and K^+ via ion channels results in the outflow of water and a decrease in cell volume. The VSOR anion channel has been implicated as the channel primarily responsible for mediating the efflux of Cl^- in this process. The channel also has a critical role in apoptosis. Activation of the channel is an early stage event required for the cell to undergo apoptotic volume decrease (AVD), which is essential for progression of the apoptotic program (Maeno et al., 2000; Okada et al., 2001; Shimizu et al., 2004). Blockage of VSOR chloride current by channel blockers inhibits AVD and apoptosis (Shimizu et al., 2004; Takahashi et al., 2005).

The molecular identity of the VSOR chloride channel is unknown, although many attempts have been made to clone the channel. Molecules proposed as candidates for the channel have included pICln (Paulmichl et al., 1992), P-glycoprotein (Valverde et al., 1992) and ClC-3 (Duan et al., 1997). These proteins may be regulators of the VSOR chloride channel but have been shown to

be distinct from the channel protein itself (Tominaga et al., 1995; Voets et al., 1996; Gong et al., 2004).

The mechanism by which the VSOR chloride channel is activated is complex and differs depending on cell type. Channel activation requires binding of intracellular ATP and can be blocked by free intracellular Mg^{2+} (Oiki et al., 1994). Regulation by tyrosine kinases and protein kinase Cα has been observed in some cell types (Abdullaev et al., 2003; Browe & Baumgarten, 2003; Chou et al., 1998; Hermoso et al., 2004). A basal level of cytosolic Ca^{2+} is thought to be required for the channel's activity (Szücs et al., 1996), and stimulation of a Ca^{2+}-sensing receptor by extracellular Ca^{2+} is known to cause upregulation of channel current (Shimizu et al., 2000). It has been shown that during apoptosis, an increase in reactive oxygen species (ROS) activates the channel (Shimizu et al., 2004). Other modulatory elements include the actin cytoskeleton, Rho monomeric G proteins and caveolin-1 (Okada, 1997; Carton et al., 2002; Trouet et al., 2001).

The main purpose of this study was to elucidate the role of functional expression of the VSOR chloride channel in cisplatin resistance. In KB/CP4 cells, expression of the VSOR chloride channel molecule itself may be downregulated, or the protein may be mislocalized. Alternatively, one or more components of the activation mechanism may be missing or defective. Initially, an attempt was made to determine the molecular identity of the channel or a regulator of it. Using a subtractive hybridization method and semi-quantitative RT-PCR, genes expressed at a higher level in KB cells than in KB/CP4 cells were identified. None of these genes appeared to be genes encoding proteins whose expression could restore VSOR chloride channel function in KB/CP4 cells, however.

Histone deacetylase (HDAC) inhibitors have been shown to induce growth arrest, differentiation and apoptosis of various tumor cells (Marks et al., 2000) and are now known to be a novel class of relatively specific anticancer agents

(Johnstone, 2002). Trichostatin A (TSA), which was originally developed as an antifungal agent, is a potent inhibitor of HDAC activity at nanomolar concentrations (Yoshida et al., 1990; Marks et al., 2001). Recently, TSA has been shown to sensitize tumor cells which are resistant to a variety of apoptotic inducers to TRAIL-mediated (Inoue et al., 2002; Sonnemann et al., 2005), anticancer drug-induced (Roh et al., 2004) and UV-induced (Kim et al., 2005) apoptosis. Thus, there is a possibility that TSA restores sensitivity to cisplatin or VSOR chloride current function in cisplatin-resistant KB carcinoma cells. In the present study, in fact, it was demonstrated that VSOR chloride current could be partially restored in KB/CP4 cells by treatment of the cells with TSA. The restoration of the current in these cells provided a means of examining the role of the channel in cisplatin resistance.

Materials and Methods

Cell culture and drug treatment

The cisplatin-resistant cell line KB/CP4 was derived from human KB epidermoid cancer cells (Murakami et al., 2001). The cells were cultured in Eagle's MEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum and 23.3 μM cisplatin (Sigma, St. Louis, MO). KB cells were cultured in medium of the same composition that did not contain cisplatin. For treatment with drugs for cell viability measurements or caspase-3 assays, KB/CP4 cells were plated at a density of ~1 x 10^4 cells/well in 96-well plates or ~2 x 10^5 cells/well in 6-well plates, respectively, two days before treatment. The medium was then replaced with Eagle's MEM containing 10% fetal bovine serum and various combinations of 400 nM trichostatin A (TSA; Wako, Osaka, Japan), 23.3 μM cisplatin and 50 μM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS; Sigma). Ethanol (Wako) and dimethylsulfoxide (Dojindo, Mashiki, Japan) were used in place of TSA and DIDS, respectively, in negative controls. For cell viability measurements, the drug-containing medium was replaced after 24 hr and the cells were incubated for 24 hr further, for a total treatment time of 48 hr before beginning assays. For caspase-3 activity assays, protein was extracted from the cells after 24 hr of treatment. For total RNA extraction, cells were plated at a density of ~3 x 10^5 cells/well in 6-well plates. RNA was extracted when the cells reached ~75% confluency. For patch-clamp experiments with TSA, cells were plated at a density of ~2 x 10^5 cells/well in 6-well plates two days before treatment; medium was then replaced with medium containing 400 nM TSA and incubated for 24 hr before experiments. For patch-clamp experiments with 5-aza-2'-deoxycytidine (Aza-dC; MP Biomedicals, Irvine, CA), cells were plated at a density of ~1.5 x 10^5 cells/well in 6-well plates two days before treatment; medium was then replaced with medium containing 2.5 μM Aza-dC and

incubated for 2 days, after which the cells were passaged. Fresh Aza-dC was added each day for 2 more days before electrophysiology experiments were performed.

Subtractive hybridization

Subtractive hybridization of cDNA derived from KB cells and KB/CP4 cells was carried out with the PCR-Select™ cDNA Subtraction Kit (Clontech, Mountain View, CA). In this method, the "tester" population of cDNAs contains the differentially expressed sequence(s) of interest while the "driver" population of cDNAs is used as a reference. cDNA sequences expressed at a significantly higher level in the tester population are specifically amplified by PCR, whereas amplification of cDNA sequences with similar expression levels in both groups is minimized. In the experiments described here, KB cDNA served as the tester and KB/CP4 cDNA as the driver. To summarize briefly, double-stranded cDNA from both cell types was digested with *Rsa I*, a restriction enzyme that generates blunt-ended fragments. The digested tester population was divided into two groups and different adaptors were ligated to the ends of molecules in each group. Each tester group was hybridized separately with an excess of driver cDNA, which was not ligated with adaptors, and both groups were then mixed together for a second hybridization in the presence of a large excess of driver cDNA. After filling in recessed ends of the molecules by DNA polymerase, PCR cycling using a single primer annealing to identical sequences in the different adaptors was used to amplify differentially expressed sequences. Using nested primers, a second round of PCR cycling was performed to further enrich the product with differentially expressed sequences and reduce background. A subtracted cDNA library was generated by ligating secondary PCR products into the pGEM-T Easy Vector (Promega, Madison, WI), electroporating ligation products into ElectroMAX™ DH5α-E™ *E.coli* cells (Invitrogen) using a Gene Pulser II with

Pulse Controller PLUS (Bio-Rad, Hercules, CA) and plating the cells onto agar plates containing ampicillin and reagents for blue-white selection.

cDNA for subtractive hybridization was generated according to the manufacturer's instructions, using messenger RNA from both cell lines. Messenger RNA was isolated from total RNA extracted using Sepasol reagent (Nacalai Tesque, Kyoto, Japan). Isolation of messenger RNA was carried out using the MPG Direct mRNA Purification Kit (CPG, Lincoln Park, NJ) with a slightly modified protocol: 50 mM NaCl was added to total RNA preps to facilitate binding of poly A+ RNA to beads.

cDNA array screening

Screening of the subtracted library was carried out with the PCR-Select™ Differential Screening Kit (Clontech), using a protocol modified to be non-radioactive. Hybridization probes were made by biotinylating secondary PCR products from the forward subtraction in a reaction containing biotin-16-dUTP (Roche Diagnostics, Penzberg, Germany) in place of radioactive nucleotides. Random clones were selected from the subtracted cDNA library and colony PCR of the clones was used to generate DNA which was arrayed on positively charged nylon membranes after denaturation with 0.6 N NaOH. This was followed by neutralization of membranes and overnight hybridization with probes at 72°C. Membranes were washed several times with a 2X SSC / 0.5% SDS buffer and 0.2X SSC / 0.5% SDS buffer at 68°C before visualization.

Arrays were visualized with an enhanced chemiluminescence method in which streptavidin-horseradish peroxidase binds to biotinylated probes and catalyzes an oxidation reaction that emits light (Non-Rad Detection Kit, BD Biosciences, San Jose, CA). Colonies showing clear hybridization signals were chosen for sequencing.

Semi-quantitative RT-PCR

For cDNA generation, 1 μg KB total RNA or 1 μg KB/CP4 total RNA was first combined in a PCR tube with 0.5 μg oligo$(dT)_{12-18}$ primer (Invitrogen) and diethylpyrocarbonate-treated (DEPC-treated) H_2O to a total volume of 10 μl, denatured at 70°C for 10 min and rapidly cooled to 4°C on a GeneAmp System 9600 thermal cycler (Perkin-Elmer, Wellesley, MA). 10 μl of a reverse transcription reaction mix containing 200 U of M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega), 500 μM dNTPs (Takara Bio, Otsu, Japan), 40 U RNase inhibitor (TOYOBO, Osaka, Japan), and 2X M-MLV Reverse Transcriptase Reaction Buffer (Promega) were then added to the denatured RNA for a total volume of 20 μl. The reverse transcription reaction was carried out by an incubation at 42°C for 50 min. This was followed by an incubation at 70°C for 15 min to inactivate the enzyme and cooling of the sample to 4°C. For negative controls, the reverse transcriptase was replaced with DEPC-treated H_2O.

PCR amplification of sequences from synthesized cDNA was carried out with a GeneAmp System 9600 thermal cycler using primers designed with Primer3 web software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and purchased from Operon Biotechnologies (Tokyo, Japan). PCR reaction mixtures consisted of 1 μl of product from reverse transcription reactions, 1X Blend Taq buffer (TOYOBO), 8 μM dNTPs, 2 μM each of forward and reverse primers, 1.25 U Blend Taq polymerase (TOYOBO) and H_2O to bring the total reaction volume to 50 μl. The standard PCR cycling protocol consisted of a 4 min denaturation step at 94°C; n cycles of a 1 min denaturation step at 94°C, a 1 min annealing step at 55°C and a 1 min extension step at 72°C; and a final hold at 4°C. The annealing temperature was increased in some cases for optimal amplification of the target sequence. PCR cycling was paused at the extension step of specified cycles and 5 μl aliquots were removed for analysis. The total

number of PCR cycles was adjusted for different targets and ranged from 28 to 43. The 5 µl aliquots were mixed with 1 µl of 6X DNA loading buffer (TAKARA) and electrophoresed on a 2% agarose/TAE gel which was then stained with a 1:10000 dilution of SYBR Gold nucleic acid stain (Molecular Probes, Eugene, OR) in TAE buffer.

Cloning of target gene open reading frames and heterologous expression

Gene open reading frames were cloned by RT-PCR. Reverse transcription of total RNA was performed as described for semi-quantitative RT-PCR. PCR was also performed as described for semi-quantitative RT-PCR, except that Advantage 2 Polymerase Mix and Advantage 2 PCR buffer (Clontech) were used in place of Blend Taq polymerase and Blend Taq buffer, respectively. The Advantage 2 Polymerase Mix contains a Taq polymerase, antibodies for automatic hot start and a proofreading polymerase for greater accuracy. Primers flanking the open reading frame were designed with Primer3 web software and purchased from Operon Biotechnologies. PCR products were cloned by T/A cloning. They were first purified with the Wizard SV Gel and PCR Clean-Up System (Promega) and then ligated into the pGEM-T Easy Vector (Promega). Competent high *E.coli* DH5α cells (TOYOBO) were transformed by heat shock with ligated plasmids and plated onto agar plates containing ampicillin for selection of transformants.

Gene sequences were subcloned into the pIRES2-EGFP mammalian expression vector (Clontech) by digesting both the gene-containing plasmids and the vector with appropriate restriction enzymes, dephosphorylating the vector, and ligating the gene-containing fragment into the vector. The pIRES2-EGFP vector allows translation of both the gene of interest and enhanced green fluorescent protein from a single bicistronic mRNA.

The Wizard Plus SV Minipreps DNA Purification System (Promega) was used for plasmid minipreparations.

Sequences were verified by dideoxynucleotide terminator sequencing with the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 or 3100 Genetic Analyzer (Applied Biosystems).

Cells were transfected with expression constructs using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Assays were carried out 24-48 hours after transfection.

RNA interference

Stealth™ RNAi molecules targeted to GPI-specific phospholipase D1 were designed with the BLOCK-iT™ RNAi Designer (https://rnaidesigner.invitrogen.com/rnaiexpress/) and purchased from Invitrogen. The sequence of the sense strand of the siRNA used was 5'-ACCAGGAUGCGUAUCAGGCUGGAAU-3'. KB cells seeded in 6-well plates at a density of 7.5 x 10^4 cells / well were transfected with RNAi molecules one day after plating using Lipofectamine 2000 reagent according to the manufacturer's instructions. BLOCK-iT™ fluorescent oligos (Invitrogen) were used for negative controls in electrophysiology experiments and to check transfection efficiency. RT-PCR and electrophysiology experiments were carried out 36-48 hr after transfection. RT-PCR to confirm knockdown of the target mRNA was performed as described for semi-quantitative RT-PCR, except that aliquots were not removed from reactions during PCR cycling.

Electrophysiology

Whole-cell patch-clamp recordings were performed as reported previously (Kubo and Okada, 1992; Liu et al., 1998). The patch electrodes were fabricated from borosilicate glass capillaries (outer diameter=1.4 mm, inner diameter=1.0 mm, Asahi Rika-Glass Industry, Nagoya, Japan) using a micropipette puller (P-2000, Sutter Instruments, Novato, CA). The wide-tipped electrodes had a resistance of around 2 MΩ when filled with pipette solution. Series resistance (<5 MΩ) was compensated (to 70-80%) to minimize voltage errors. Currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Current signals were filtered at 1 kHz using a four-pole Bessel filter and digitized at 4 kHz. pClamp software (version 6.0.2; Axon Instruments) was used for command pulse control, data acquisition and analysis. The time course of current activation and recovery was monitored by repetitively applying (every 15 s) alternating step pulses (2 s duration) from a holding potential of 0 mV to ±40 mV. To observe voltage dependence of the current profile, and inactivation kinetics at large positive potentials in particular, step pulses (2 s duration) were applied from a prepotential of -100 mV to test potentials of -80 to +100 or +120 mV in 20-mV increments after attaining steady activation of swelling-induced current. The amplitude of instantaneous current was measured at 1.25 ms after the step pulse onset.

The control isotonic external solution contained (in mM): 110 CsCl, 5 $MgSO_4$, 12 HEPES, 7 Tris, and 100 mannitol (pH 7.5, 330 mosmol kg-H_2O^{-1}). The control hypotonic solution was made by reducing the concentration of mannitol to 20 mM (pH 7.5, 250 mosmol kg-H_2O^{-1}). The osmolality of solutions was measured using a freezing-point depression osmometer (OM802, Vogel, Germany). The pipette (intracellular) solution contained (in mM): 110 CsCl, 2 $MgSO_4$, 0.4 $CaSO_4$, 1 Na_2-ATP, 15 Na-HEPES, 10 HEPES, 1 ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), and 50 mannitol (pH

7.3, 300 mosmol kg-H_2O^{-1}; pCa 7). Na_2-ATP was omitted from the solution in experiments to test the intracellular ATP dependency of currents. The osmolality of the pipette solution was set lower (by 30 mosmol kg-H_2O^{-1}) than that of the control isotonic bathing solution in order to prevent spontaneous cell swelling after attaining the whole-cell mode (due to poorly diffusible cytosolic constituents: Worrell et al., 1989).

Cell volume measurements

The mean cell volume was measured at room temperature using a Coulter counter (CDA-500, Sysmex, Japan), as reported previously (Hazama and Okada, 1988). The mean volume of the cell population was calculated from the cell volume distribution after the machine was calibrated with latex beads of known volume. Isotonic or hypotonic solution consisted of (in mM): 95 NaCl, 4.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 110 or 0 mannitol and 5 HEPES-NaOH (pH 7.3; 310 or 200 mosmol kg-H_2O^{-1}).

Cell viability assay

Cell viability of cells plated in 96-well plates was assessed by measuring mitochondrial succinate dehydrogenase activity, using the Cell Counting Kit-8 (Dojindo). This kit utilizes WST-8, a tetrazolium derivative which serves as a substrate for the enzyme and releases a water-soluble formazan compound upon reduction by the enzyme. The amount of formazan produced gives a measure of cell viability. Formazan production 1 hour after addition of 10 μl substrate to each well was quantified by the absorbance at 414 nm, using a Multiskan MS-UV absorbance spectrophotometer (Thermo Electron, Waltham, MA).

Caspase-3 activity assay

Caspase-3 activity in extracts from drug-treated KB/CP4 cells was measured using the CaspACE Assay System, Fluorometric (Promega). In the assay, cleavage of N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin, a substrate for caspase-3, releases 7-amino-4-methylcoumarin, which produces a blue fluorescence upon excitation. Cell extracts were incubated with the substrate in the presence and absence of an inhibitor specific to caspase-3 so that fluorescence due to cleavage of the substrate by caspase-3, and not by non-specific proteases, could be calculated. An Fmax fluorescence plate reader (Molecular Devices, Sunnyvale, CA) with a 355 nm excitation / 460 nm emission filter was used to quantify fluorescence.

To prepare cell extracts, cells were pelleted by a 5 min centrifugation at 600 x *g*, washed with PBS, lysed in a hypotonic lysis buffer (25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, 2 mM PMSF, 10 μg/ml pepstatin A and 10 μg leupeptin) and subjected to three freeze-thaw cycles. After a 20 min centrifugation at 15000 x *g*, the supernatant was removed for use in the caspase assay. Samples were kept on ice throughout the extraction process. The amount of protein in each sample was quantified by the Bio-Rad Protein Assay (Bio-Rad) so that caspase activity values could be normalized.

Stock solutions

Drugs were diluted from stock solutions to final concentrations during experiments. A 100 mM stock of DIDS was prepared in DMSO; a 100 mM stock of phloretin in DMSO; a 5 mM stock of trichostatin A in ethanol; a 25 mM stock of Aza-dC in 50% acetic acid; a 500 mM stock of 1,10-phenanthroline (Sigma) in ethanol; and a 1.165 mM stock of cisplatin in Eagle's MEM. Stock solutions were stored at -30°C, except for cisplatin, which was stored at 4°C.

Statistics

Values presented are means ± S.E.M. of n observations. Data were evaluated by the unpaired or paired Student's t-test and a P value of less than 0.05 was taken to indicate statistical significance.

Results

Identification of mRNAs differentially expressed in KB and KB/CP4 cells

mRNAs differentially expressed in KB and KB/CP4 cells were identified. First, subtractive hybridization of cDNA derived from KB cells (tester) and KB/CP4 cells (driver) was performed and a forward-subtracted cDNA library was obtained. Forward-subtracted probes were generated using the same subtractive hybridization products. Of 960 clones randomly chosen from the library and screened, 75 clones were selected for sequencing based on their hybridization with forward-subtracted probes on membrane arrays. A list of genes sequenced is shown in Table 1. Some sequences appeared multiple times, most notably, that for lactate dehydrogenase A (LDHA), which represented 30 of the 75 clones sequenced. The reason this gene appeared so many times is unclear. Also, it is noted that argininosuccinate synthetase (ASS; splice variant 2), thymosin β4 X-linked (TMSB4X) and S100 calcium binding protein A4 (S100A4) appeared three, three and two times, respectively. To confirm the differential expression of transcripts identified, semi-quantitative RT-PCR was performed for selected sequences, using cDNA derived from the two cell lines. Some genes identified by other means than subtractive hybridization, namely, folate receptor 1 (FOLR1) and GPI-specific phospholipase D (GPLD1), were also checked. FOLR1 was selected because of a report that its expression was downregulated in a cisplatin-resistant KB cell line (Higuchi et al., 2003). GPLD1 was chosen because of a report that phospholipase D is involved in the activation of the VSOR chloride channel (Tomassen et al., 2004). Seven genes were found to be expressed at higher levels in KB cells than in KB/CP4 cells (Fig. 1), while other genes did not show clear differences in expression level (Fig. 2).

Overexpression of full-length cDNAs in KB/CP4 cells

Overexpression experiments were carried out to determine whether the genes found to be differentially expressed are involved in regulation of VSOR chloride current. First, full-length open reading frames for selected genes were cloned by RT-PCR and T/A cloning, and then subcloned into a mammalian expression vector (pIRES2-EGFP). The expression constructs were transfected into KB/CP4 cells and 24-48 hr later, cells expressing GFP fluorescence were patch-clamped for assay of VSOR chloride channel activity. Overexpression of full-length cDNAs of LDHA, S100A4, TMSB4X, FOLR1, GPLD1 (splice variant 2) and ASS (splice variant 2) did not result in restoration of volume-sensitive chloride current in KB/CP4 cells (Fig. 3). Because of difficulties in cloning the full-length cDNA of ALPP by PCR, its overexpression in KB/CP4 cells was not tested.

Knockdown of GPI-specific phospholipase D1 in KB cells

It was postulated that GPLD1, one of the genes identified as a differentially expressed gene, might be a regulator of volume-sensitive chloride current, based on a report that phospholipase D has a role in the activation of the current (Tomassen et al., 2004). The effects of siRNA-mediated silencing of GPLD1 transcripts in KB cells were therefore observed. Transfection of cells with siRNA reduced the amount of both transcript variants 1 and 2 of GPLD1 (Fig. 4A). Whole-cell patch-clamp showed an increase in volume-sensitive chloride current for siRNA-transfected cells (Fig. 4B). This was an unexpected result because transcript variant 2 of GPLD1 was found to be expressed at a higher level in KB cells, not KB/CP4 cells (Fig. 1), and GPLD1 transcript variant 1 did not have a clear difference in expression level (data not shown). It is possible

that GPLD1 downregulates volume-sensitive chloride current but it is unlikely to be responsible for the absence of the current in KB/CP4 cells.

Phenanthroline effects on regulatory volume decrease

1,10-phenanthroline, a chelator of heavy metal ions, is known to inhibit GPI-specific phospholipase D1 (Low and Prasad, 1988). In order to further test whether GPLD1 is involved in regulation of volume-sensitive chloride current, the effect of phenanthroline on regulatory volume decrease in KB cells was observed. Inhibiting a component essential for the activation of volume-sensitive chloride current would be expected to inhibit regulatory volume decrease, while blocking an inhibitor of the current might facilitate it. It was found that KB cells incubated for 30 min in medium containing 0.5 mM phenanthroline underwent a much more rapid regulatory volume decrease after hypotonic stimulation than cells receiving the control treatment (Fig. 5). A similar acceleration of RVD was observed in HeLa cells treated with phenanthroline, suggesting a shared phenanthroline-sensitive component in the mechanism of RVD in KB and HeLa cells. Whole-cell recordings made of currents in KB cells treated with phenanthroline showed that there was no alteration in volume-sensitive chloride currents, however (data not shown).

The fact that phenanthroline facilitated, rather than inhibited, regulatory volume decrease in KB and HeLa cells suggests that GPLD1 may be an inhibitor of RVD. However, phenanthroline treatment does not appear to enhance VSOR chloride current, suggesting that it does not remove inhibition of the VSOR chloride channel. An alternative explanation for the acceleration of RVD by phenanthroline is that the chemical may have an effect on proteins other than GPLD. It is known that proteins involved in cellular energy metabolism, for example, are modulated by phenanthroline (Gerber et al., 1996).

Restoration of VSOR current expression in TSA-treated KB/CP4 cells

Because overexpression of selected genes did not result in restoration of VSOR chloride current in KB/CP4 cells, a different strategy for restoring expression of the current was adopted. Besides gene overexpression, removal of gene inhibition due to DNA methylation or histone deacetylation could potentially result in restoration of volume-sensitive chloride current, so drugs which are known to remove inhibition of gene expression were tested. Treating cells for 96 hr with 2.5 μM Aza-dC, a DNA methyltransferase inhibitor, did not result in restoration of VSOR chloride current (Fig. 6). However, treatment for more than 24 hr with 400 nM TSA, a drug which inhibits histone deacetylases, caused a significant restoration of whole-cell volume-sensitive chloride current (Fig. 7). Without ATP present in the pipette solution, the current could not be activated by TSA (Fig. 8). The TSA-induced current was blocked in a voltage-dependent manner by DIDS (Fig. 9) and in a voltage-independent manner by phloretin (Fig. 10). These pharmacological properties, along with the dependency of the current on intracellular ATP, are strong evidence that the current induced is classical VSOR chloride current.

Effects of TSA on cell volume regulation

VSOR current activation is essential for regulatory volume decrease in most cell types (Okada, 1997). Because treatment with TSA results in partial restoration of VSOR chloride current in KB/CP4 cells, it might be expected that volume regulation in these cells would also be affected by TSA treatment. In comparison to KB cells, KB/CP4 cells respond to hypotonic stimulation with a very slow, weak regulatory volume decrease (Ise et al., 2002). To see whether this RVD would be facilitated by pre-treatment of cells with TSA, the volume response of TSA-treated KB/CP4 cells to stimulation with a 65% hypo-osmotic

solution was measured. It was found that RVD was only slightly enhanced, after about 35 min of hypotonic stimulation (Fig. 11). The reason that RVD was not facilitated to a greater degree may be, in addition to the fact that VSOR chloride current restoration was only partial, that other elements of the RVD mechanism may be impaired in this cell type.

Morphological changes in TSA-treated KB/CP4 cells

KB/CP4 cells treated with TSA showed prominent morphological changes. Phase-contrast photomicrographs were taken 12, 24 and 36 h after treatment with TSA. By 24 h of treatment, cells had developed an elongated, star-like appearance, and processes extended from some of them (Fig. 12). Cells also appeared to aggregate to a lesser degree.

Changes in gene expression levels after TSA treatment

24-h TSA treatment of KB/CP4 cells resulted in the upregulation of certain genes and the downregulation of others. Of the genes expressed at a higher level in KB cells than in KB/CP4 cells, FOLR1 and GPLD1 (splice variant 2), were found to be upregulated in KB/CP4 cells after TSA treatment, whereas LDHA, S100A4, TMSB4X and ASS were not affected by the treatment (Fig. 13A). ALPP also was not affected by TSA treatment (data not shown). Of genes checked because of their role in apoptosis, TNFR1 was found to be downregulated after TSA treatment (Fig. 13B), while TNFR2, TNFα and Fas did not appear to be expressed in these cells. These results are in contrast to previous reports that TSA induces upregulation of expression of several death receptors and their ligands in lymphoma (Nakata et al., 2004) and leukemia (Insinga et al., 2005) cells. Caveolin-1 expression was checked because caveolin-1 is known to be necessary for activation of the VSOR chloride channel in calf pulmonary

artery endothelial cells (Trouet et al., 2001). However, caveolin-1 expression appeared to decrease, rather than increase, after TSA treatment (Fig. 13B).

Decrease in cisplatin resistance of KB/CP4 cells treated with TSA and blockage of the decrease by a VSOR chloride channel blocker

Cell viability measurements were performed to determine whether trichostatin A treatment affects the cisplatin resistance of KB/CP4 cells. It was found that treatment for 48 h with trichostatin A alone caused a significant decrease in cell viability, as measured by mitochondrial succinate dehydrogenase activity. However, cell viability decreased further in cells treated with 23.3 μM cisplatin (CP4) in addition to trichostatin A (TSA), suggesting that the cells' sensitivity to cisplatin increased (Fig. 14). To determine whether VSOR chloride channel activity might account for this increased sensitivity, 50 μM DIDS, a blocker of the channel, was added along with trichostatin A and cisplatin. DIDS appeared to block the increase in cisplatin sensitivity, suggesting that the VSOR chloride channel does in fact play a role in the cisplatin sensitivity of these trichostatin A-treated cells.

Cisplatin-induced apoptosis in TSA-treated KB/CP4 cells

To determine whether the cisplatin-induced viability decrease of TSA-treated KB/CP4 cells was due, at least in part, to apoptosis, caspase-3 activity in these cells was measured. Cells treated for 24 h with TSA and cisplatin (CP4) had a significantly enhanced level of caspase-3 activity in comparison to cells treated with TSA alone (Fig. 15). This enhancement was blocked by addition of 50 μM DIDS, suggesting that the increase in apoptosis was dependent on activity of the VSOR chloride channel.

Discussion

Cisplatin is an effective chemotherapeutic agent used for the treatment of a variety of human cancers (Rozencweig et al., 1977; Loehrer and Einhorn, 1984). One of the major limitations to the use of these drugs is the acquisition of resistance by initially responsive tumor cells (Loehrer and Einhorn, 1984; Ozols, 1992; Perez et al., 1993). Cisplatin resistance is thought to involve several mechanisms, such as inactivation of cisplatin by thiol compounds (Nakagawa et al., 1988; Saburi et al., 1989), reduced cisplatin accumulation (Hromas et al., 1987; Waud, 1987; Teicher et al., 1987; Kraker and Moore, 1988; Kuppen et al., 1988; Mann et al., 1990; Rixe et al., 1996; Fujii et al., 1994) by an actively pumping transporter other than P-glycoprotein (Fujii et al., 1994) or MRP3 (Uchiumi et al., 1998), accelerated DNA repair (Behrens et al., 1987; Eastman and Schulte, 1988; Lai et al., 1988; Masuda et al., 1988; Sheibani et al., 1989; Chaney and Sancar, 1996; Husain et al., 1998), increased metallothionein (Kelley et al., 1988; Kasahara et al., 1991), a defect in the signal transduction pathway of the EGF receptor (Christen et al., 1990) and defective endosomal acidification (Chauhan et al., 2003). However, the mechanisms of cisplatin resistance have not been firmly determined. In the present study, I have demonstrated that cisplatin resistance is associated with an impairment of VSOR chloride channel function.

How are cisplatin resistance and the disruption of VSOR chloride current linked? An answer to this in the most general terms is that an alteration of gene expression is likely to be involved. The activity of the channel may be disrupted by changes in gene expression which result in the alteration of proteins critical to the activation process of, or the proper trafficking of, the channel. There are many reports of differential gene expression in cisplatin resistance. Transcription factors (Torigoe et al., 2005), a vacuolar proton pump gene (Murakami et al., 2001), and a copper-transporting P-type ATPase (Komatsu et al., 2000) are

among genes that are upregulated. Transporters such as FOLR1 and genes involved in endocytic pathways and protein trafficking such as small GTPases have reduced expression (Shen et al., 2004). Mislocalization of some membrane proteins related to multidrug resistance in cisplatin-resistant cancer cell lines (Liang et al., 2003) might be a factor in disruption of VSOR current expression; this mislocalization may also be due to altered gene expression.

It is also conceivable that cisplatin alters some proteins by direct binding. For example, cisplatin is known to bind a copper transporter, hCtr1, and stabilize a multimer of this protein (Guo et al., 2004). However, it seems less likely that VSOR chloride current downregulation is due to a direct effect of cisplatin on a protein, because it seems to be more of a long-term effect in KB/CP4 cells which are cultured with cisplatin; short-term treatment of non-resistant KB cells with cisplatin actually potentiates the current (Ise et al., 2002).

Also, it is known that plasma membrane fluidity can be altered by cisplatin (Lacour et al., 2004; Dimanche-Boitrel et al., 2005). Such fluidity changes might also affect activation of the VSOR chloride channel; it was recently shown that changes in the amount of cholesterol can modulate, but not abolish, the activity of the channel in endothelial cells (Romanenko et al., 2004). Thus, it appears that a cisplatin-induced membrane fluidity increase may affect activation of the VSOR chloride channel, but may not account for disruption of this channel associated with cisplatin resistance.

The purpose of this study was to try to probe the relationship between cisplatin resistance and VSOR chloride channel activity—are the two linked by a change in gene expression? If it is in fact a change in gene expression then it might be possible to identify the channel molecule, or a regulator, by a subtractive hybridization method. So initially, subtractive hybridization to identify differentially expressed genes was carried out, and how VSOR chloride current was affected by overexpressing or inhibiting differentially expressed genes was observed.

Differential expression of genes in KB and KB/CP4 cells

Differential expression of certain genes in KB and KB/CP4 cells was evident from the results of semi-quantitative RT-PCR (Fig. 1). That the expression of a variety of genes is altered in cisplatin resistance is not an unexpected result. Of interest is what the consequences of these alterations are. Are the differentially expressed genes involved in cisplatin resistance, in VSOR chloride channel activity or both? Do specific genes serve as a common link between both channel activity and the cells' response to cisplatin?

Overexpression of selected genes expressed at higher levels in KB cells did not result in restoration of VSOR chloride current expression in KB/CP4 cells. How overexpression of differentially expressed genes affects cisplatin resistance was not checked because of the low efficiency of transfection into KB/CP4 cells (<5%). Cisplatin resistance results from a multitude of factors (Wang & Lippard, 2005; Wernyj & Morin, 2004) and it is conceivable that the lack of VSOR chloride current expression also results from more than a single change. If it is the case that multiple parts of the activation mechanism are impaired, for example, or that more than one protein required for proper trafficking are obstructed, then overexpression of a single gene in KB/CP4 cells would not be sufficient to restore expression of VSOR chloride current.

On the other hand, knockdown of a factor essential to the mechanism of channel activation would be sufficient to block channel activation in cells normally expressing the channel. This was attempted using siRNA for GPI-specific phospholipase D1 in KB cells. However, the results failed to meet my expectations. Knockdown did not result in reduction of channel activity, and surprisingly, resulted in an enhancement of volume-sensitive chloride current (Fig. 4), suggesting that GPLD1 might function to downregulate, rather than upregulate, the current.

Inhibition of GPLD1 by a blocker was also attempted. Treatment by phenanthroline had no effect on VSOR currents but did affect RVD. This was seen in both KB and HeLa cells (Fig. 5). It is known that phenanthroline is a chelator of heavy metal ions such as iron (Gerber et al., 1996), so it is possible that the effect on RVD may be due not to its effect on GPI-specific phospholipase D1, but to effects on other proteins which require heavy metal ions.

Partial restoration of VSOR by trichostatin A

Making the assumption that changes in gene expression are responsible for the absence of VSOR chloride current, and specifically, that inhibition of gene expression is taking place, it makes sense to try removing gene inhibition as one strategy for attempting to restore VSOR expression. Epigenetic changes such as promoter hypermethylation and histone deacetylation are known to be responsible for generalized gene inhibition (Jones and Baylin, 2002; Johnstone, 2002).

Gene inhibition by hypermethylation can be reversed by treatment with Aza-dC, a DNA methyltransferase inhibitor. In one study, hypermethylation of the folate receptor gene in a cisplatin-resistant KB cell line was seen to be reversed by Aza-dC (Shen et al., 2004). In the current study, however, activation of folate receptor gene transcription was not seen in Aza-dC-treated KB/CP4 cells (data not shown), and upregulation of VSOR chloride current expression was not observed either (Fig. 6).

Acetylation and deacetylation of histones change higher order chromatin structure by altering histone interactions with DNA (Lee et al., 1993; Wolffe and Guschin, 2000). The level of histone acetylation, which determines the transcriptional status of chromatin, is regulated by a dynamic equilibrium between histone acetyltransferases (HATs) and histone deacetylases (HDACs)

(Weidle and Grossmann, 2000; Narlikar et al., 2002). Histone deacetylation acts in favor of gene silencing in concert with histone methylation (Nakayama et al., 2001). Gene inhibition by histone deacetylation can be reversed by histone deacetylase inhibitors. Trichostatin A, an HDAC inhibitor, had a marked effect on VSOR chloride current expression in KB/CP4 cells (Fig. 7). Without treatment, the current was virtually absent but with treatment the current could be activated, although not to the same level as in KB cells. This effect is likely to be mediated by an alteration of gene expression, because of the well-known restorative effect of TSA on aberrantly silenced genes. TSA is known to both upregulate and downregulate the expression of genes. In pancreatic adenocarcinoma cells, for example, it caused both the induction and repression of genes, although the number of upregulated genes was greater (Moore et al., 2004). Trichostatin A inhibits HDACs at nanomolar concentrations (Yoshida et al., 1990), presumably by chelating a zinc ion in the active site pocket of the HDAC enzyme through its hydroxamic acid group (Marks et al., 2001). It has been reported to induce cell cycle arrest, apoptosis and differentiation in cancer cells (Marks et al., 2000). It causes a net acetylation of histone proteins and chromatin decondensation, and its effects are reversible. Because of its ability to act specifically on cancer cells, its potential as a drug for cancer treatment is being investigated (Marks et al., 2001; Johnstone, 2002).

Why was the restoration of VSOR chloride current in KB/CP4 cells a partial restoration? Partial restoration of the current may be a reflection of the restoration of expression of a limited number of genes. The expression of all genes which were found to be expressed at lower levels in KB/CP4 cells than in KB cells was not restored (Fig. 13A). The genes found to be upregulated by TSA treatment appeared to be a subset of the genes which were downregulated in KB/CP4 cells. Alternatively, partial restoration of the current may be due to the partial restoration of expression of a single gene; folate receptor expression, for

example, appeared to be partially--not fully--restored (compare Fig. 1 with Fig. 13A).

Treatment of KB/CP4 cells with trichostatin A appeared to affect regulatory volume decrease to only a very small degree (Fig. 11). This may be due to the fact that VSOR chloride current restoration was not complete. Another reason is that other elements of the RVD mechanism may be impaired in this cell type and the restoration of volume-sensitive chloride current may not be sufficient for restoration of RVD. The mechanism of RVD is known to be very complex, involving many elements including nonselective cation channels, potassium channels and calcium and ATP receptors (Okada et al., 2001). Some cell types express VSOR chloride current but do not undergo RVD (De Smet et al., 1994).

What is the mechanism by which VSOR is activated in these TSA-treated cells? Morphological changes may be responsible for the induction of VSOR current expression, because the VSOR anion channel, and specifically its volume sensitivity, is known to be regulated by the cytoskeleton (Okada, 1997). Clear morphological changes were observed (Fig. 12). In human carcinoma cells, morphological changes caused by TSA treatment were found to be the result of de novo gene expression and protein synthesis induced by the treatment (Hoshikawa et al., 1994).

Decrease in cisplatin resistance by trichostatin A treatment, enhanced apoptosis and the role of VSOR chloride current

A question that naturally arises from the finding that volume-sensitive chloride current is induced by TSA in cisplatin-resistant KB cells is whether the cells also lose their cisplatin resistance because of this treatment. If so, the question of whether this decrease is related to the phenotypic change in VSOR chloride current expression follows. As shown by cell viability measurements in TSA- and cisplatin-treated KB/CP4 cells, cisplatin resistance does in fact decrease (Fig.

14). Treatment with a VSOR chloride channel blocker, DIDS, prevented this decrease in cell viability, suggesting that the decrease in cisplatin resistance is a result of increased VSOR chloride current expression.

What is the mechanism by which TSA decreases the cisplatin resistance of KB/CP4 cells, causing a decrease in cell viability? It has been proposed that a more open configuration of chromatin resulting from the inhibition of histone deacetylases facilitates the interaction of anticancer drugs with DNA or enzymes that act upon DNA, increasing the cytotoxicity of these drugs (Kim et al., 2003). This may be part of the mechanism by which cisplatin sensitivity increases in KB/CP4 cells. Also, the acetylation of non-histone proteins that results from inhibition of HDACs cannot be ruled out as a factor in the effects of TSA (Henderson and Brancolini, 2003). The present study suggests that the induction of VSOR chloride current by TSA treatment, which may be the result of alterations in gene expression, is part of the mechanism. VSOR chloride current expression has been shown to be essential for the induction of apoptosis (Maeno et al., 2000; Shimizu et al., 2004). Since VSOR is upregulated in TSA-treated KB/CP4 cells, it seems quite plausible that an increase in apoptosis permitted by the upregulation of channel activity could account for the decrease in cell viability of TSA-treated cells exposed to cisplatin. Indeed, apoptosis, as measured by caspase-3 activity, was found to increase in TSA-treated KB/CP4 cells exposed to cisplatin, compared to cells receiving TSA treatment alone (Fig. 15). Furthermore, addition of a VSOR chloride channel blocker, DIDS, prevented this increase in apoptosis.

HDAC inhibitors were shown to induce apoptosis of tumor cells by themselves (Marks et al., 2000) and to sensitize anticancer drug-resistant tumor cells to antitumor agent-induced apoptosis (Roh et al., 2004). Recent studies have listed possible mechanisms for the HDAC inhibitor effects on tumor cells, such as downregulation of P-glycoprotein in multidrug resistant tumor cells (Castro-Galache et al., 2003), downregulation of antiapoptotic Bcl-2 family members

(Bcl-2 and Bcl-XL) together with upregulation of an apoptotic member (Bad) (Sawa et al., 2001; Mitsiades et al., 2004; Yee et al., 2004; Duan et al., 2005) and upregulation of death receptors and their ligands (Nakata et al., 2004; Nebbioso et al., 2005; Insinga et al., 2005; Sutheesophon et al., 2005). Here, I have provided an additional mechanism; that is, upregulation of VSOR chloride channel activity, which is known to have an essential role in induction of apoptosis (Shimizu et al., 2004).

Thus, TSA treatment, by causing functional upregulation of the VSOR chloride channel, decreased the resistance of KB/CP4 cells to cisplatin. This suggests the clinical utility of HDAC inhibitors like TSA which, while having anticancer activity on their own, could, in combination with anticancer drugs like cisplatin, be the basis for cancer treatment that is more effective than that possible with conventional anticancer drugs alone. The simultaneous use of an HDAC inhibitor like TSA with cisplatin would reduce the resistance of cancer cells resistant to the drug and thus result in increased cancer cell killing by cisplatin. The increased killing of cancer cells by anticancer drugs in combination with HDAC inhibitors has been reported previously (Kim et al., 2003). The major worry in the clinical use of any drug, however, is the unintended side effects resulting from action of the drug on other targets--in this case, normal, non-cancerous cells of the body. HDAC inhibitors seem to be toxic to cancer cells specifically, although the basis of this specificity is not understood (Johnstone and Licht, 2003). In animals, hydroxamic acid-based HDAC inhibitors, the class of HDAC inhibitors to which TSA belongs, have been shown to limit tumor cell growth with little or no toxicity (Marks et al., 2001). Furthermore, many phase I clinical trials indicate that HDAC inhibitors appear to be well-tolerated drugs (Villar-Garea and Esteller, 2004). So far, HDAC inhibitors appear to be very promising new drugs for the treatment of cancer.

In conclusion, although the identity of the VSOR chloride channel remains unknown, it is clear that malfunction of the channel can contribute to the cisplatin resistance of cancer cells. This study shows that restoration of volume-sensitive chloride current in a cisplatin-resistant KB cell line enhances sensitivity to cisplatin by permitting an increase in apoptosis. It also suggests the utility of histone deacetylase inhibitors like trichostatin A in reducing the resistance of cancer cells to treatment with anticancer agents, and of targeting the VSOR chloride channel for upregulation in cancer therapy.

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Figure legends

Figure 1. Genes downregulated in cisplatin-resistant KB (KB/CP4) cells.
The expression level of selected genes in KB and KB/CP4 cells was compared by semi-quantitative RT-PCR. LDHA, S100A4, TMSB4X, ASS and ALPP were identified by subtractive hybridization of cDNA from the two cell types. The number of cycles at which samples were taken, and PCR product sizes in base pairs (bp), are indicated. (-RT), negative controls in which reverse transcriptase was excluded from the cDNA synthesis reaction. LDHA, lactate dehydrogenase A; S100A4, S100 calcium binding protein A4; TMSB4X, thymosin, beta 4, X-linked; ASS, argininosuccinate synthetase; ALPP, alkaline phosphatase, placental; FOLR1, folate receptor 1; GPLD1 var2, GPI-specific phospholipase D1, transcript variant 2; Markers, 100 bp DNA ladder.

Figure 2. Genes whose expression was unaltered in cisplatin-resistant KB (KB/CP4) cells.
The expression level of selected genes in KB and KB/CP4 cells was compared by semi-quantitative RT-PCR. All genes, except for PLD1, were identified by subtractive hybridization of cDNA from the two cell types. The number of cycles at which samples were taken, and PCR product sizes in base pairs (bp), are indicated. (-RT), negative controls in which reverse transcriptase was excluded from the cDNA synthesis reaction. FLJ11342, hypothetical protein FLJ11342; CALM2, calmodulin 2; TRAP1, TNF receptor-associated protein; DHCR24, 24-dehydrocholesterol reductase; RAB7, RAS oncogene family member; ITM1, integral membrane protein 1; PLD1, phospholipase D1; GAPD, glyceraldehyde-3-phosphate dehydrogenase; DDX3X, DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked; EI24, etoposide induced 2.4 mRNA; Markers, 100 bp DNA ladder.

Figure 3. Lack of enhancement of volume-sensitive chloride current in KB/CP4 cells transiently transfected with differentially expressed genes.
Volume-sensitive, outwardly rectifying chloride current was measured by whole-cell patch-clamp in KB/CP4 cells transfected with expression constructs for selected genes. Recordings were made 24-48 hours after transfection. Current-voltage relationships are presented for cells transfected with LDHA, S100A4, TMSB4X, ASS (var2: splice variant 2), FOLR1 and GPLD1 (var2: splice variant 2). Step pulses were applied from -100 mV to +100 mV in 20-mV increments. Open circles, mock-transfected cells under isotonic conditions; open triangles, mock-transfected cells under hypotonic conditions; closed circles, gene-transfected cells under isotonic conditions; and closed triangles, gene-transfected cells under hypotonic conditions. Gene abbreviations are as in figure 1.

Figure 4. Effects of siRNA-mediated silencing of GPLD1 on VSOR current in KB cells.
VSOR chloride current was measured by whole-cell patch-clamp in KB cells transfected with siRNA targeted to GPLD1. A) RT-PCR showing siRNA-mediated silencing of GPLD1, splice variants 1 (33 cycles) and 2 (38 cycles). As a control, the expression level of GAPD (18 cycles) was checked. B) Current-voltage relationships for cells transfected with siRNA for GPLD1 and control siRNA. Step pulses were applied from -100 mV to +100 mV in 20-mV increments. Open circles, mock-transfected cells under isotonic conditions; open triangles, mock-transfected cells under hypotonic conditions; closed circles, siRNA-transfected cells under isotonic conditions; and closed triangles, siRNA-transfected cells under hypotonic conditions. *Asterisks* indicate values are significantly different from mock-transfected ($P < 0.05$).

Figure 5. Effects of phenanthroline on regulatory volume decrease in KB and HeLa cells.

A) Time course of cell volume changes in response to a 65% hypo-osmotic stimulation (at *arrow*) in KB cells treated with 0.5 mM phenanthroline (PNT) for 30 min prior to stimulation. B) Time course of cell volume changes in HeLa cells receiving identical treatment. *Asterisks* indicate values are significantly different from the control ($P < 0.05$).

Figure 6. Effects of Aza-dC on VSOR chloride current in KB/CP4 cells.

VSOR chloride current was measured by whole-cell patch-clamp in KB/CP4 cells treated with and without 2.5 μM Aza-dC for 96 hours. CP4 (23.3 μM) was present in the medium throughout the treatment. A) Representative recording showing time course of currents induced by hypotonic stimulation in (a) control (Control/CP4) and (b) Aza-dC-treated (Aza-dC/CP4) cells. Currents were elicited by alternating 2-s pulses from 0 to ±40 mV. Step pulses from -100 mV to +100 mV in 20-mV increments were applied at time points indicated by *asterisks*. *Bar* above the recording indicates the time during which hypotonic solution was applied. B) Currents elicited by step pulses during application of hypotonic solution, in control and Aza-dC-treated cells. C) Current-voltage relationships for control and Aza-dC-treated cells.

Figure 7. Effects of TSA on VSOR chloride current in KB/CP4 cells.

VSOR chloride current was measured by whole-cell patch-clamp in KB/CP4 cells pretreated with and without 400 nM TSA for more than 24 hours. CP4 (23.3 μM) was present in the medium throughout the treatment. A) Representative recording showing time course of currents induced by hypotonic stimulation in (a) control (Control/CP4) and (b) TSA-treated (TSA/CP4) cells. Currents were elicited by alternating 2-s pulses from 0 to ±40 mV. Step pulses from -100 mV to +100 mV in 20-mV increments were applied at time points

indicated by *large asterisks*. *Bar* above the recording indicates the time during which hypotonic solution was applied. B) Currents elicited by step pulses during application of hypotonic solution, in control and TSA-treated cells. C) Current-voltage relationships for control and TSA-treated cells. *Small asterisks* indicate values are significantly different from the control ($P < 0.05$).

Figure 8. Effects of ATP removal on TSA-induced VSOR chloride current in KB/CP4 cells.

ATP was omitted from the pipette (intracellular) solution in whole-cell recordings of VSOR chloride current in TSA-treated KB/CP4 cells. CP4 (23.3 μM) was present in the medium throughout the TSA treatment. A) Representative recording showing time course of currents induced by hypotonic stimulation in TSA-treated cells. Currents were elicited by alternating 2-s pulses from 0 to ±40 mV. Step pulses from -100 mV to +100 mV in 20-mV increments were applied at time points indicated by *large asterisks*. *Bar* above the recording indicates the time during which hypotonic solution was applied. B) Currents elicited by step pulses during application of (a) isotonic and (b) hypotonic solution. C) Current-voltage relationships obtained from step pulses applied during hypotonic stimulation of TSA-treated cells, using pipette solution with (TSA/CP4-hypo) and without ATP (TSA/CP4-hypo, ATP-free). *Small asterisks* indicate values are significantly different from the control ($P < 0.05$).

Figure 9. Blockage of TSA-induced VSOR chloride current by DIDS in KB/CP4 cells.

The effects of DIDS on TSA-induced VSOR chloride current during hypotonic stimulation were observed by whole-cell patch-clamp. CP4 (23.3 μM) was present in the medium throughout the TSA treatment. A) Representative recording showing time course of currents induced by hypotonic stimulation in TSA-treated cells and block of currents by DIDS. Currents were elicited by

alternating 2-s pulses from 0 to ±40 mV. Step pulses from -100 mV to +100 mV in 20-mV increments were applied at time points indicated by *large asterisks*. *Bars* above the recording indicate times during which hypotonic solution and DIDS were applied. B) Currents elicited by step pulses during hypotonic stimulation (a) without and (b) with DIDS present. C) Current-voltage relationships obtained from step pulses applied before hypotonic stimulation (TSA/CP4-iso), and during stimulation of TSA-treated cells without (TSA/CP4-hypo) and with (TSA/CP4-hypo + 50 μM DIDS) DIDS present. *Small asterisks* indicate values are significantly different from the control (TSA/CP4-hypo) ($P < 0.05$).

Figure 10. Blockage of TSA-induced VSOR chloride current by phloretin in KB/CP4 cells.

The effects of phloretin on TSA-induced VSOR chloride current during hypotonic stimulation were observed by whole-cell patch-clamp. CP4 (23.3 μM) was present in the medium throughout the TSA treatment. A) Representative recording showing time course of currents induced by hypotonic stimulation in TSA-treated cells and block of currents by phloretin. Currents were elicited by alternating 2-s pulses from 0 to ±40 mV. Step pulses from -100 mV to +100 mV in 20-mV increments were applied at time points indicated by *large asterisks*. *Bars* above the recording indicate times during which hypotonic solution and phloretin were applied. B) Currents elicited by step pulses during hypotonic stimulation (a) without and (b) with phloretin present. C) Current-voltage relationships obtained from step pulses applied before hypotonic stimulation (TSA/CP4-iso), and during stimulation of TSA-treated cells without (TSA/CP4-hypo) and with (TSA/CP4-hypo + 100 μM phloretin) phloretin present. *Small asterisks* indicate values are significantly different from the control (TSA/CP4-hypo) ($P < 0.05$).

Figure 11. Effects of pretreatment with TSA on regulatory volume decrease in KB/CP4 cells.

Time course of cell volume changes in response to a 65% hypo-osmotic stimulation (at *arrow*) in KB/CP4 cells pretreated with TSA for 24 hours prior to stimulation. *Asterisks* indicate values are significantly different from the control ($P < 0.05$).

Figure 12. Morphological changes in TSA-treated KB/CP4 cells.

Phase-contrast photomicrographs of TSA-treated KB/CP4 cells were taken at 12 h, 24 h and 36 h of treatment, as indicated. *Control* cells were treated with ethanol in place of TSA for 24 h. *Scale bar* = 100 μm.

Figure 13. Gene expression in TSA-treated KB/CP4 cells.

The expression levels of selected genes in control and TSA-treated KB/CP4 cells were compared by semi-quantitative RT-PCR. A) The expression levels in TSA-treated KB/CP4 cells of genes previously identified as being expressed at a higher level in KB cells. Gene abbreviations are as in figure 1. B) The downregulation of TNF receptor 1 (TNFR1) and caveolin-1 (CAV1) gene expression levels in TSA-treated KB/CP4 cells. The number of cycles at which samples were taken, and PCR product sizes in base pairs (bp), are indicated. Markers, 100 bp DNA ladder.

Figure 14. Cell viability decrease in TSA-treated KB/CP4 cells exposed to cisplatin and blockage of the decrease by DIDS.

Cell viability was measured 48 h after treatment with 400 nM TSA, 23.3 μM cisplatin (CP4) and 50 μM DIDS in different combinations as indicated. *Control* cells were treated with ethanol in place of TSA for 48 h. n=12 per group. *Asterisk* indicates that the value is significantly different from the other groups ($P < 0.05$).

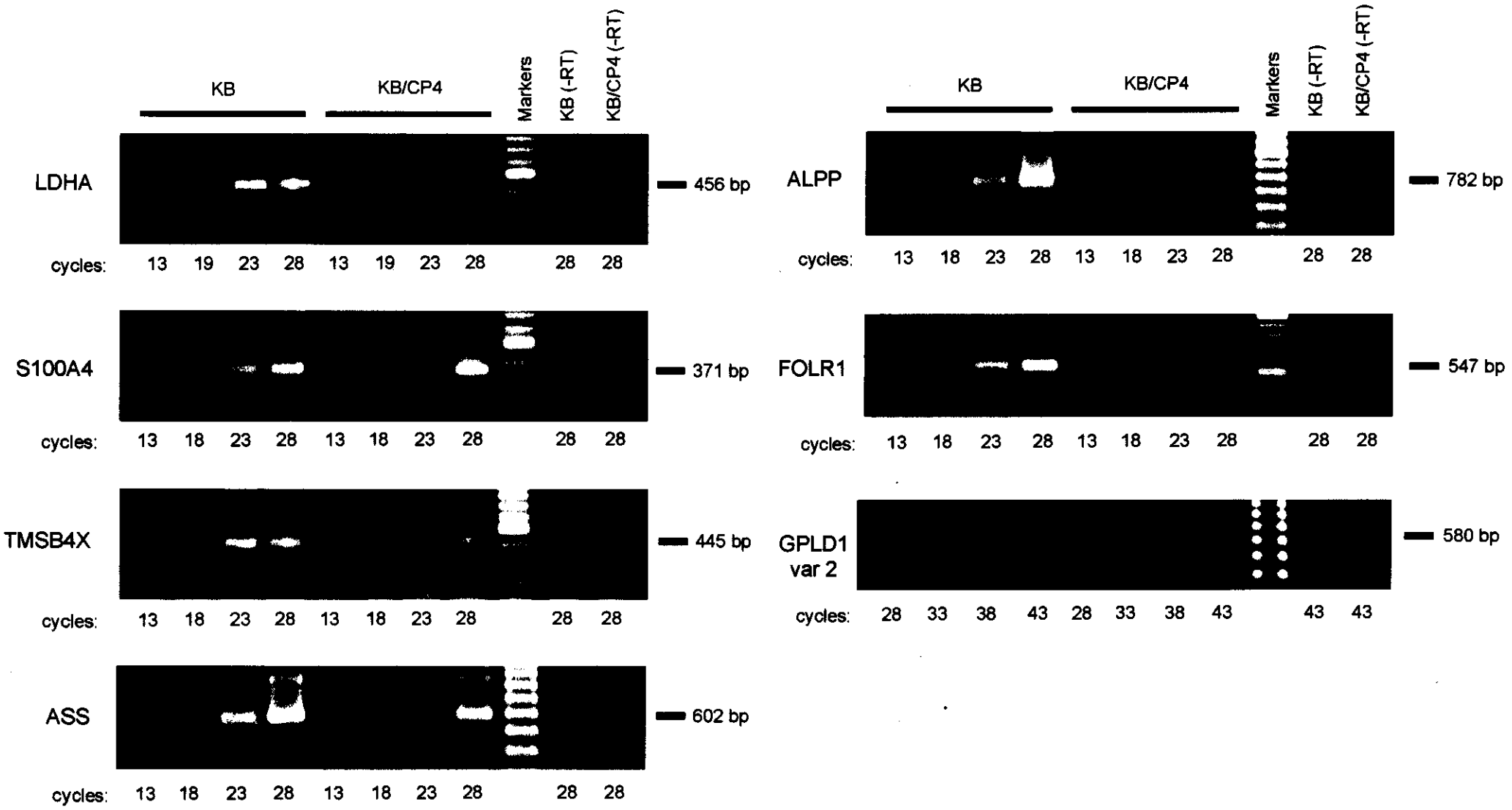
Figure 15. Caspase-3 activity increase in TSA-treated KB/CP4 cells exposed to cisplatin and blockage of the increase by DIDS.

Cell viability was measured 24 h after treatment with 400 nM TSA, 23.3 μM cisplatin (CP4) and 50 μM DIDS in different combinations as indicated. n=12 per group. *Asterisk* indicates that value is significantly different from the other groups ($P < 0.05$).

Table 1. Genes identified by subtractive hybridization

Name (var: splice variant)	GenBank accession no.	Number of clones sequenced
Argininosuccinate synthetase, var 2 (ASS)	NM_054012	3
Lactate dehydrogenase (LDHA)	NM_005566	30
S100 calcium binding protein A4 (S100A4)	NM_019554	2
Thymosin, beta4, X-linked (TMSB4X)	NM_021109	3
Actin, beta	NM_001101	3
Proteasome (prosome, macropain) 26S subunit, ATPase, 1	NM_002802	1
Hypothetical protein FLJ11342	NM_018394	1
RAB7, member RAS oncogene family	NM_004637	1
Forkhead box M1, var 1	NM_202002	1
Proteasome (prosome, macropain) subunit, beta type, 7	NM_002799	1
Alkaline phosphatase, placental (Regan isozyme) (ALPP)	NM_001632	1
Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	1
ATPase, Na^+/K^+ transporting, alpha 1 polypeptide	NM_000701	1
Protein expressed in non-metastatic cells 1, var 1	NM_198175	1
APEX nuclease (multifunctional DNA repair enzyme) 1, var 1,2 or 3	NM_001641, NM_080648 or NM_080649	2
Heat shock 60kDa protein 1 (chaperonin), nuclear gene encoding mitochondrial protein, var 1 or 2	NM_002156 or NM_199440	1
24-dehydrocholesterol reductase	NM_014762	1
TNF receptor-associated protein	NM_016292	2
Calmodulin 2 (phosphorylase kinase, delta)	NM_001743	1
Interphase cyctoplasmic foci protein 45	NM_017872	1
H63 breast cancer expressed gene (H63) var 1 or 2	NM_138423 or NM_177974	1
Heterogeneous nuclear ribonucleoprotein A/B var 1	NM_031266	1
Ferritin, light polypeptide	NM_000146	1
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked, var 2	NM_001356	1
Etoposide induced 2.4 mRNA	NM_004879	1
Integral membrane protein 1	NM_152713	1
Tropomyosin 3	NM_153649	1
Chaperonin containing TCP1, subunit 2 (beta)	NM_006431	1
Membrane interacting protein of RGS16	NM_016641	1
Bone marrow stromal cell antigen 2	NM_004335	1

Figure 1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KB/CP4
Markers
KB (-RT)
KB/CP4 (-RT)
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S100A4
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TMSB4X
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ALPP
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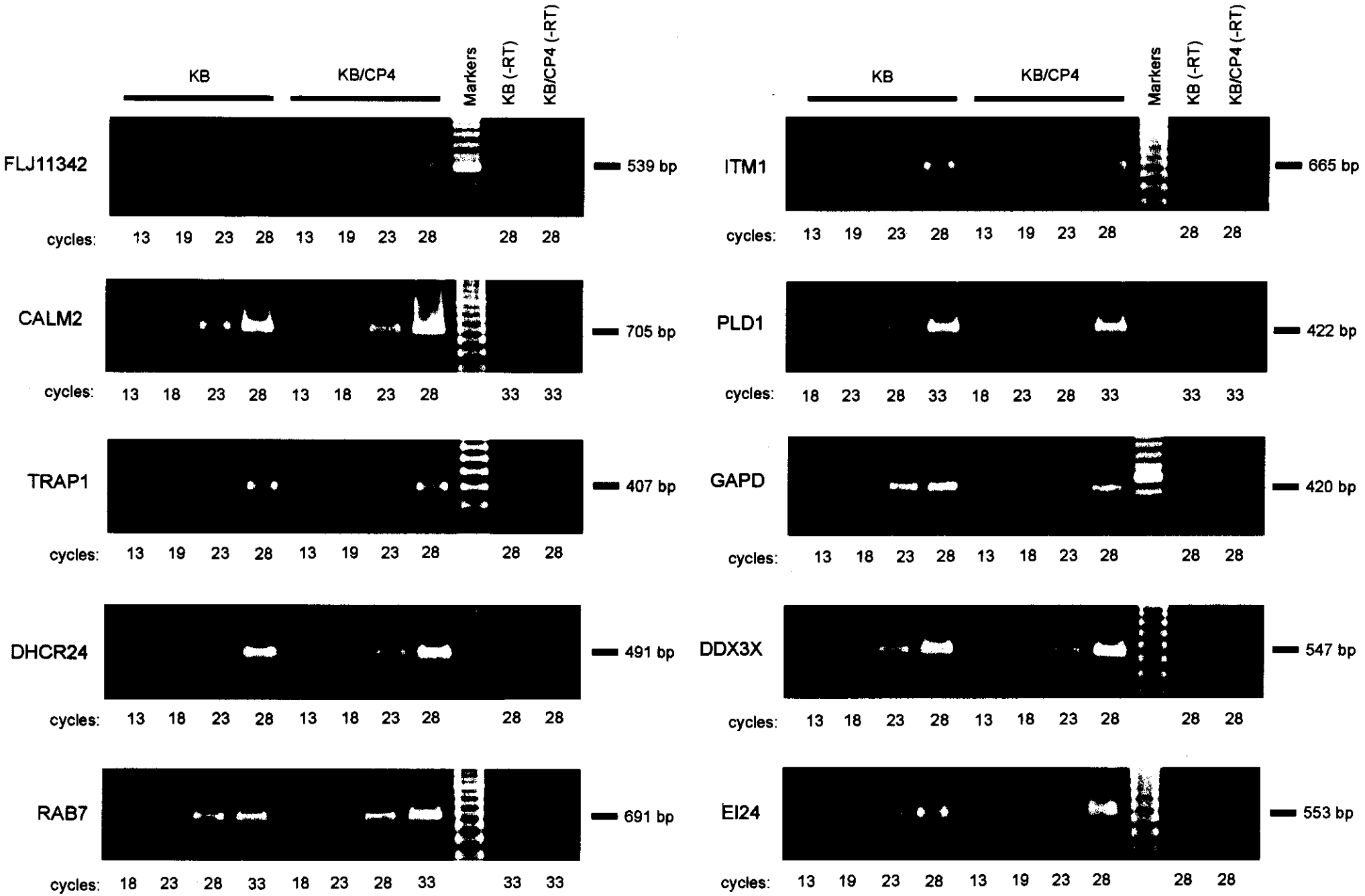
Figure 2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KB/CP4
Markers
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KB/CP4 (-RT)
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CALM2
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TRAP1
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DHCR24
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RAB7
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PLD1
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GAPD
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Figure 3

In KB/CP4 cells

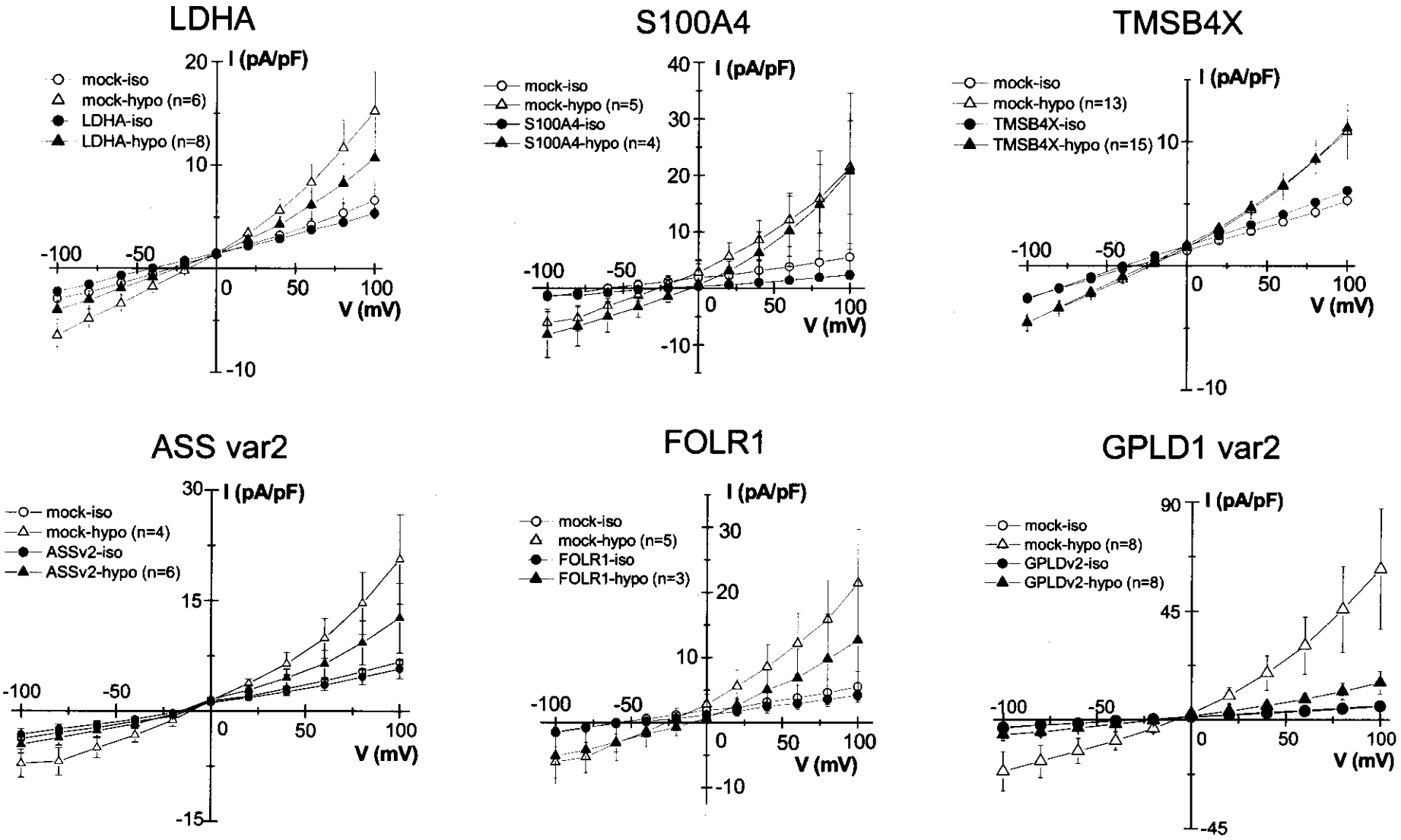
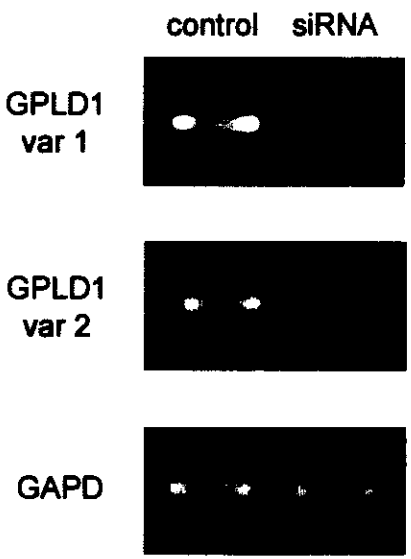


Figure 4

A


control
siRNA
GPLD1
var 1
GPLD1
var 2
GAPD

B

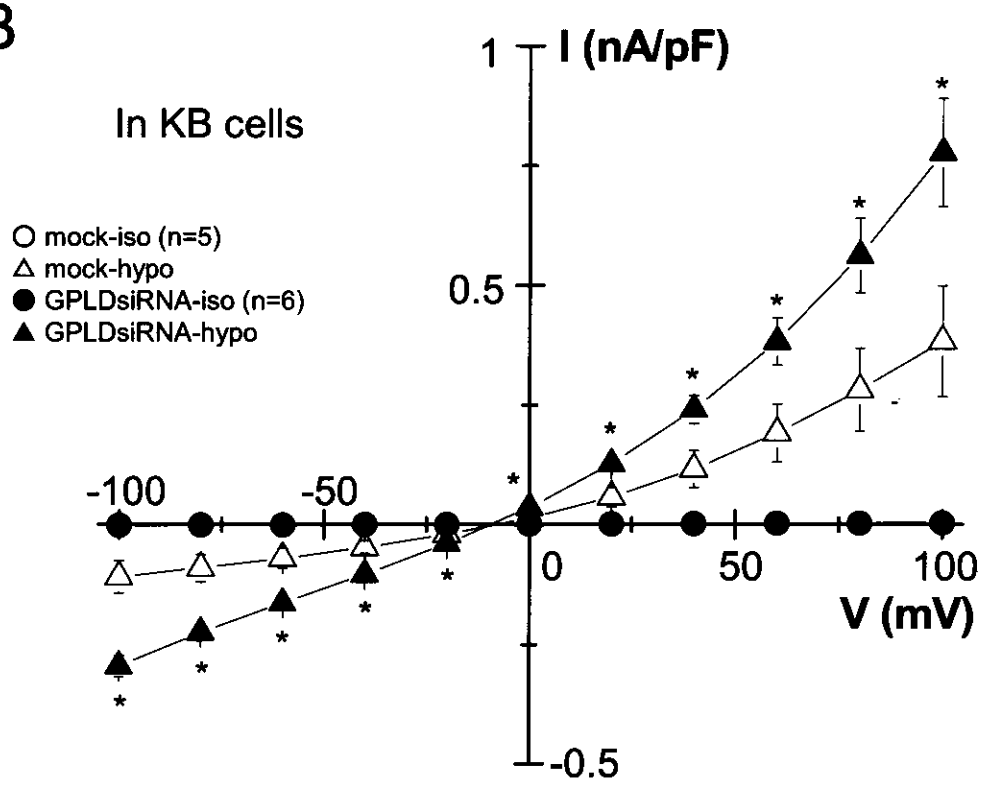
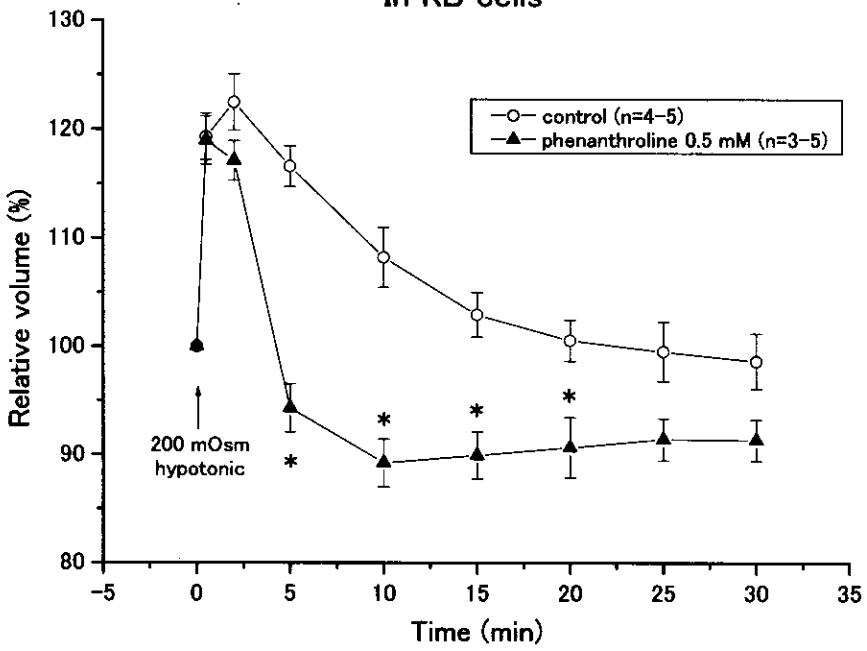

In KB cells
mock-iso (n=5)
mock-hypo
GPLDsiRNA-iso (n=6)
GPLDsiRNA-hypo
I (nA/pF)
1
0.5
-0.5
-100
-50
0
50
100
V (mV)

Figure 5

A


In KB cells
control (n=4-5)
phenanthroline 0.5 mM (n=3-5)
Relative volume (%)
130
120
110
100
90
80
200 mOsm
hypotonic
*
*
*
*
*
-5
0
5
10
15
20
25
30
35
Time (min)

B

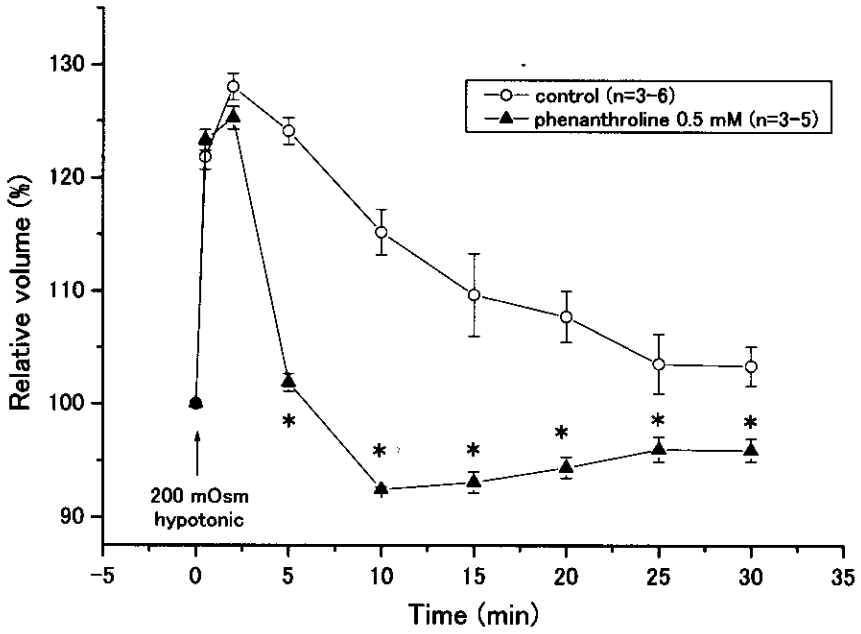

In HeLa cells
control (n=3-6)
phenanthroline 0.5 mM (n=3-5)
Relative volume (%)
130
120
110
100
90
200 mOsm
hypotonic
*
*
*
*
*
*
*
-5
0
5
10
15
20
25
30
35
Time (min)

Figure 6

In KB/CP4 cells

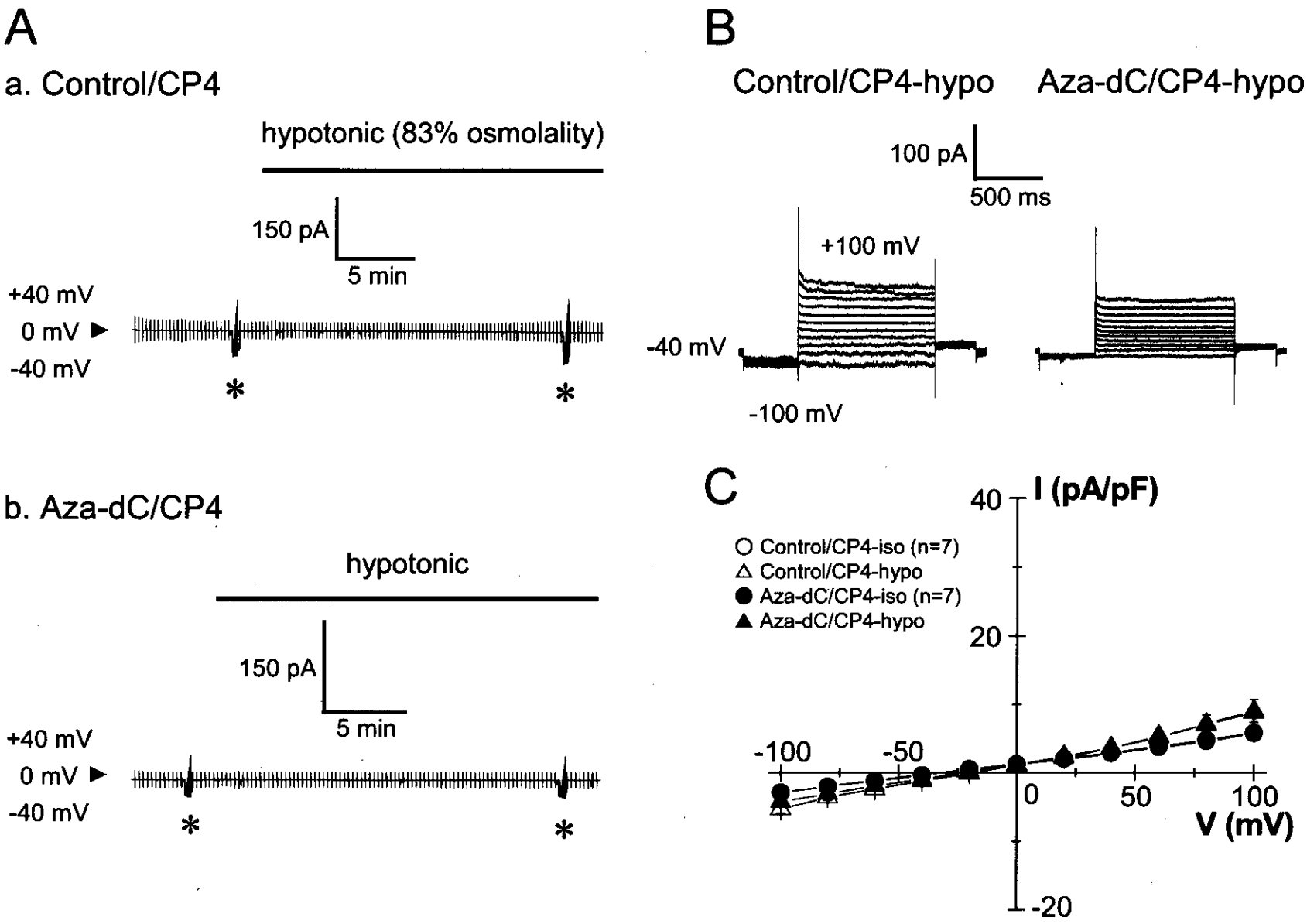
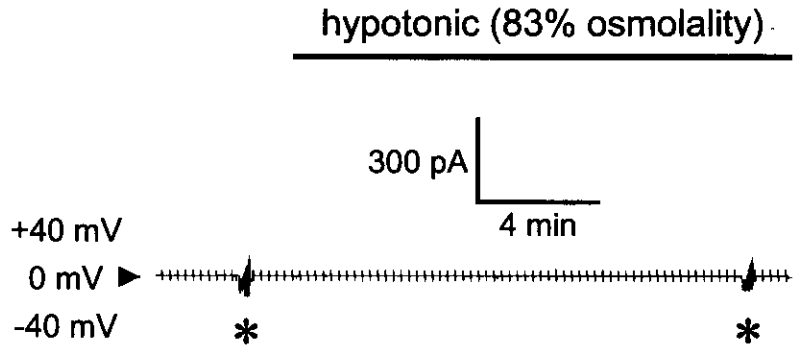


Figure 7

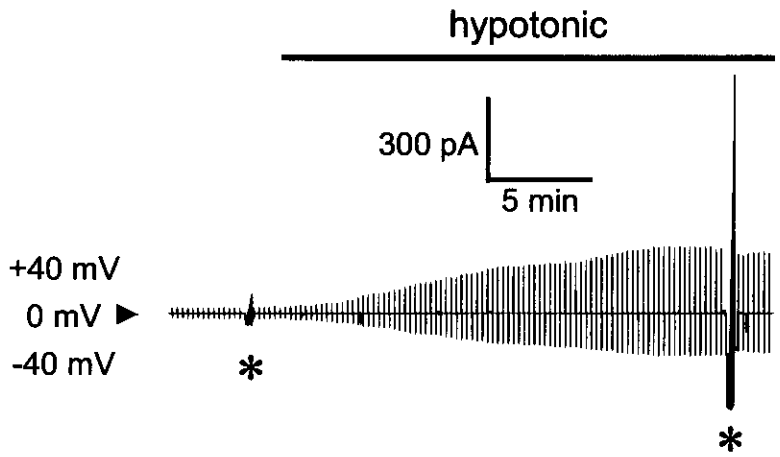
In KB/CP4 cells

A

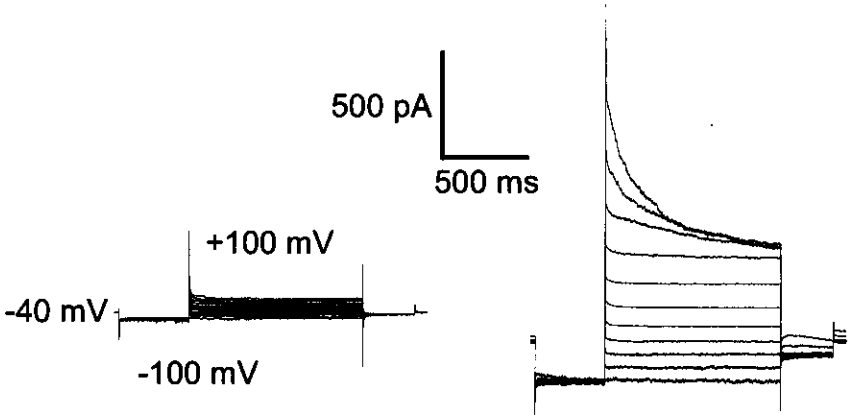
a. Control/CP4



b. TSA/CP4



B



C

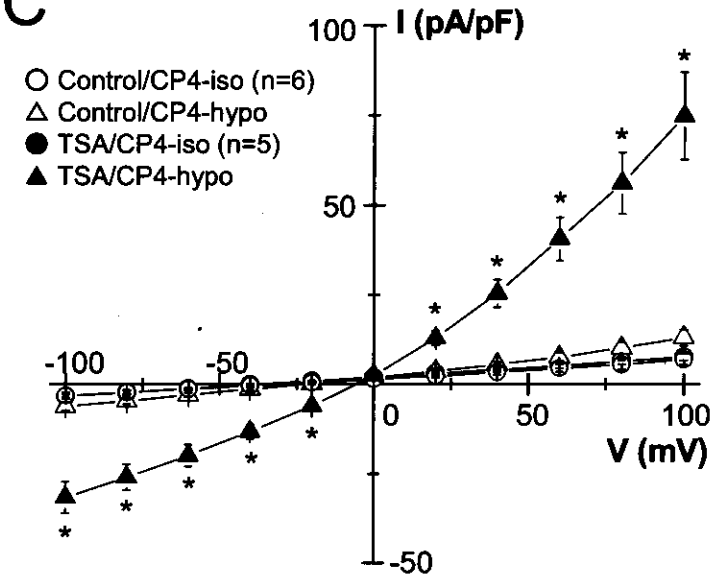


Figure 8

In KB/CP4 cells

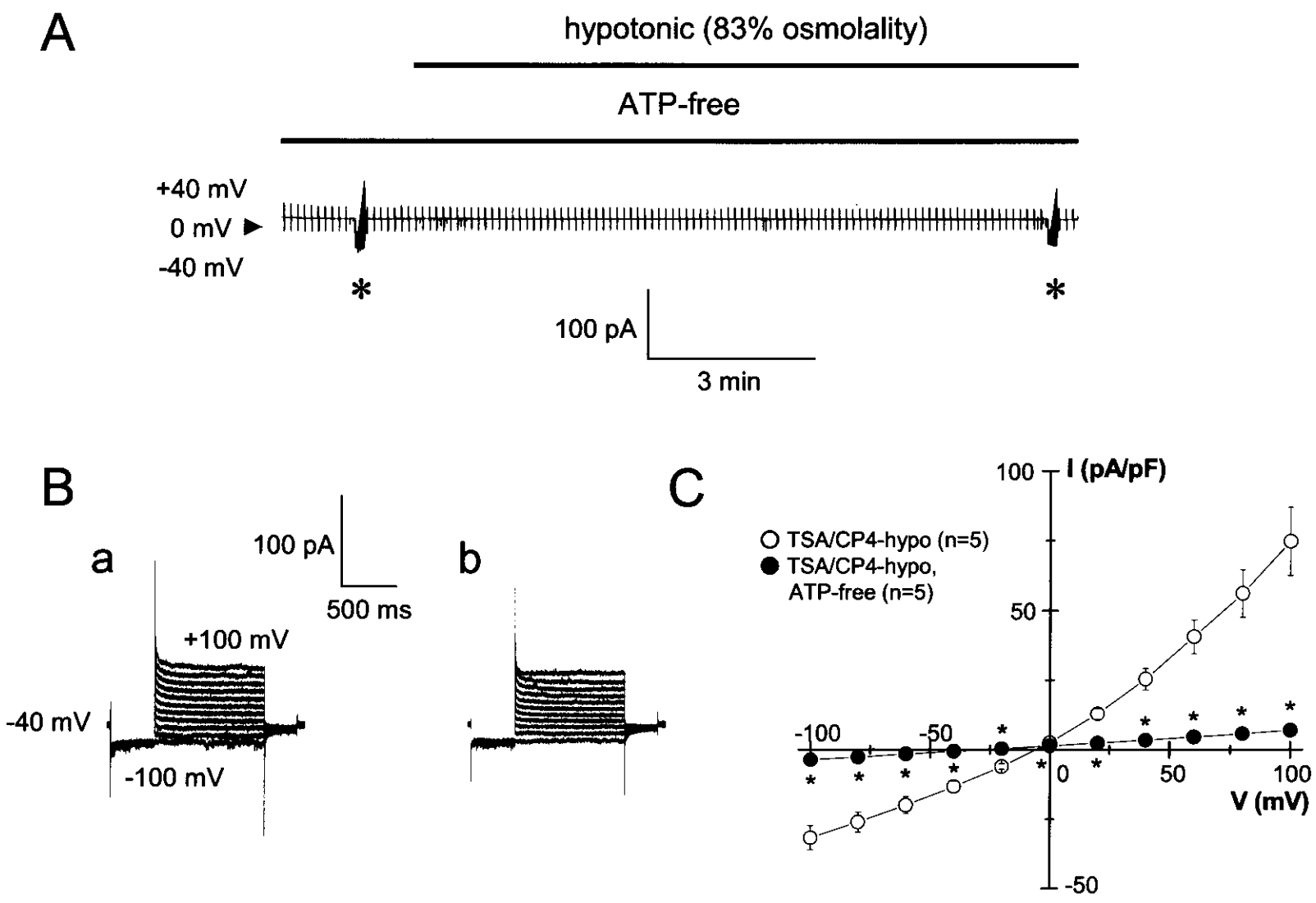


Figure 9

In KB/CP4 cells

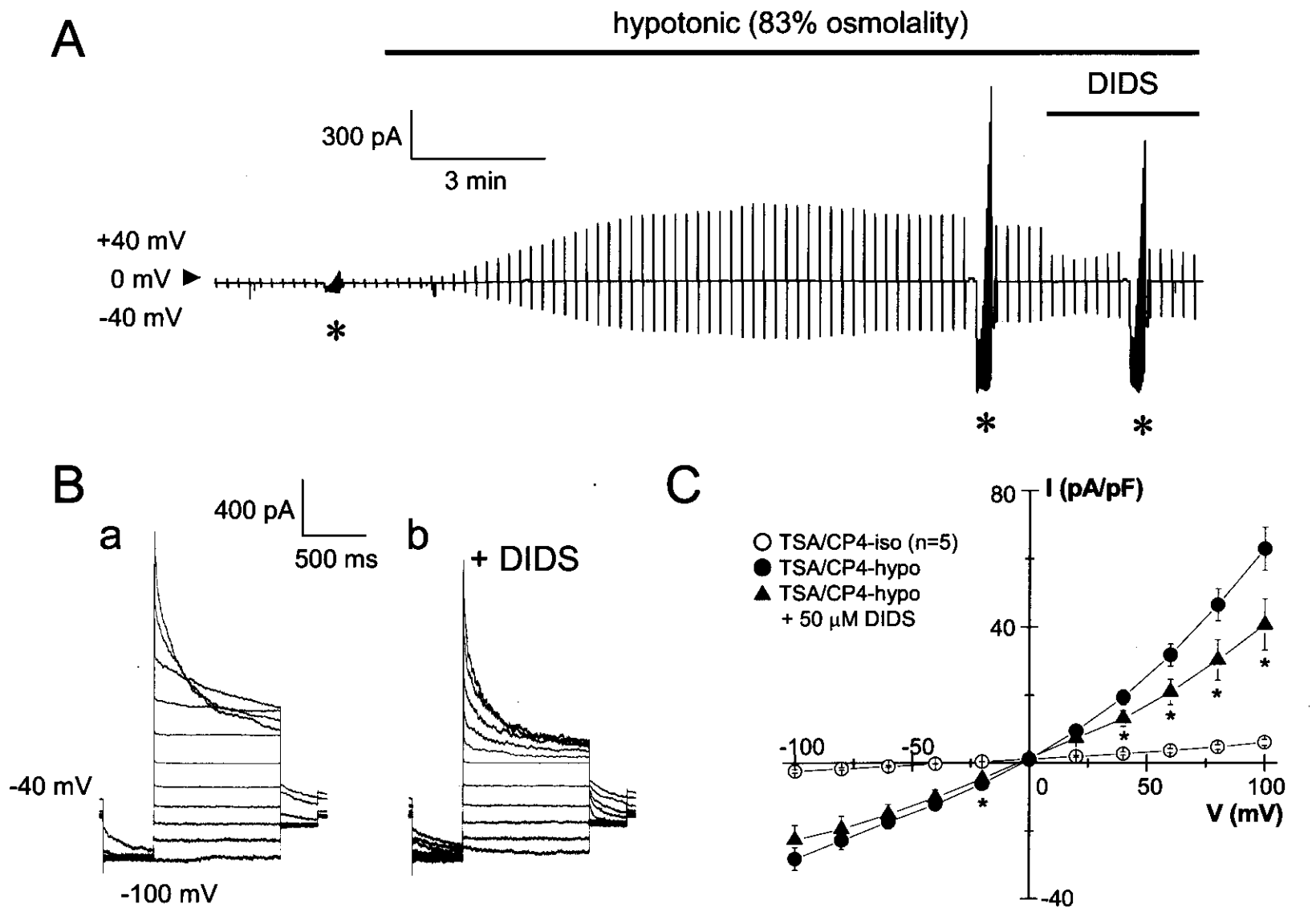


Figure 10

In KB/CP4 cells

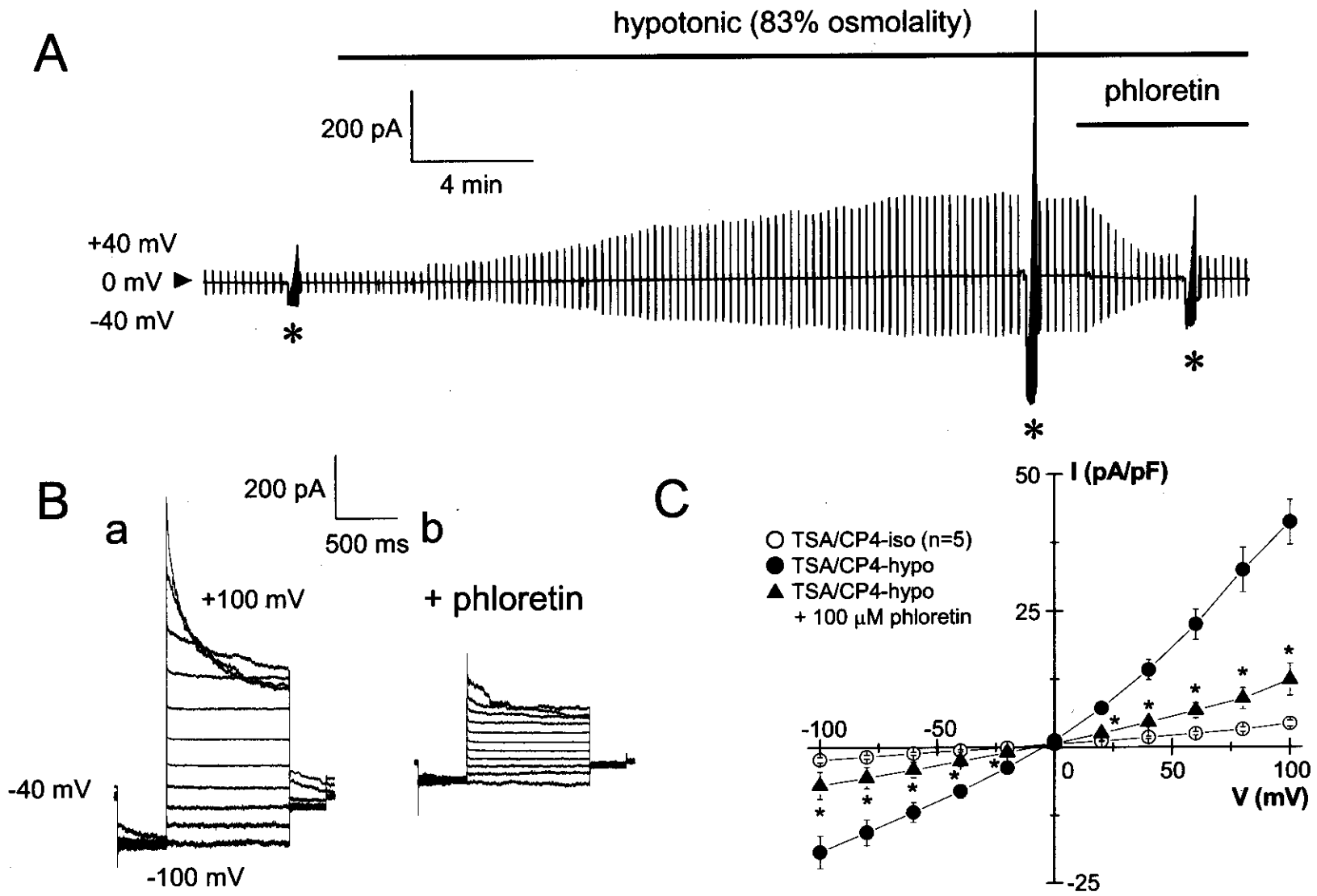


Figure 11

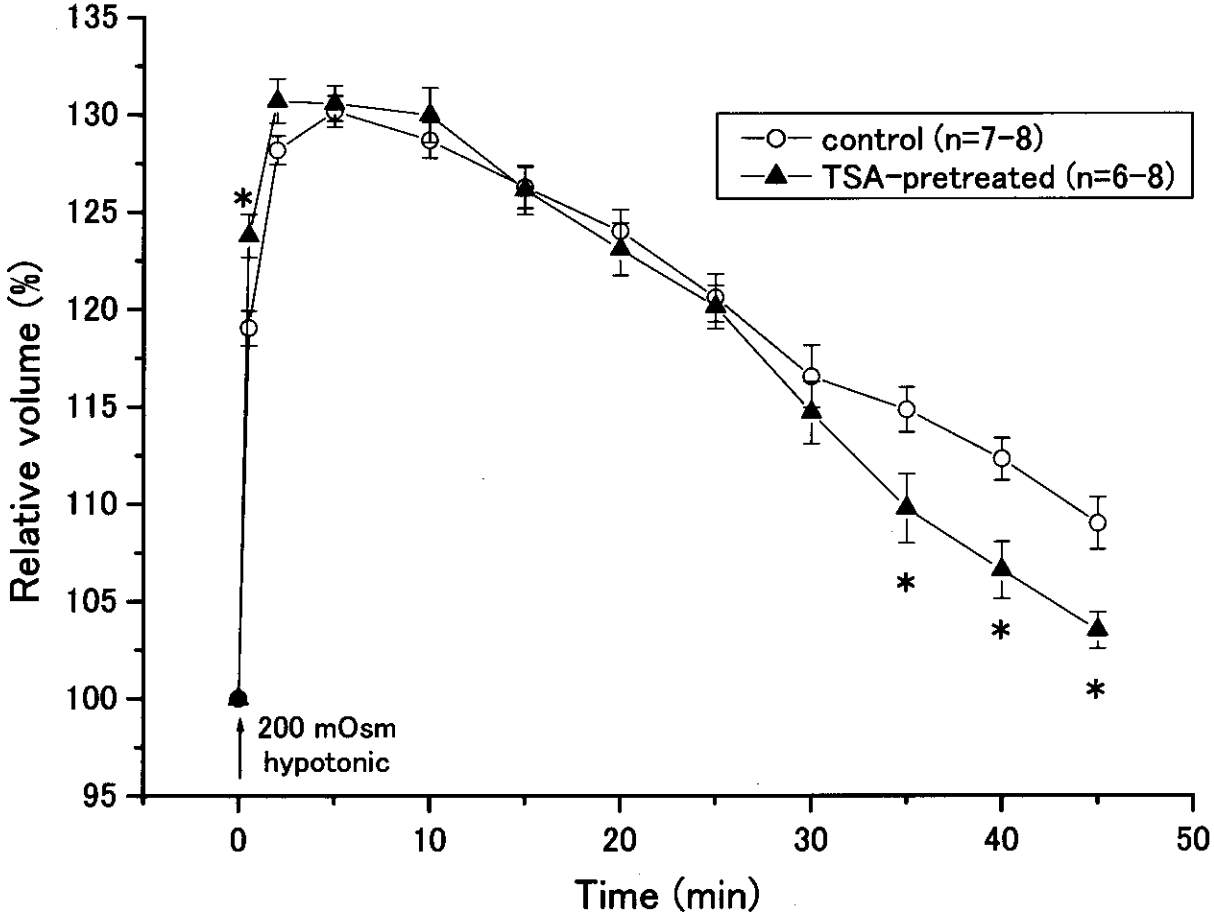
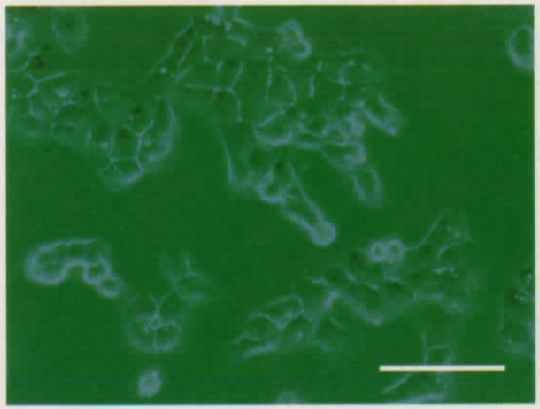

135
130
125
120
115
110
105
100
95
Relative volume (%)
0
10
20
30
40
50
Time (min)
control (n=7-8)
TSA-pretreated (n=6-8)
200 mOsm
hypotonic
*
*
*
*

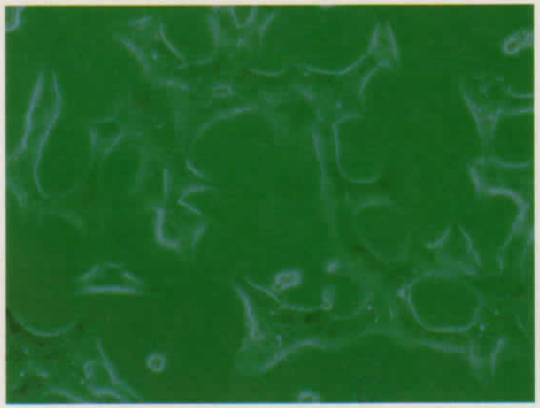
Figure 12

KB/CP4 cells

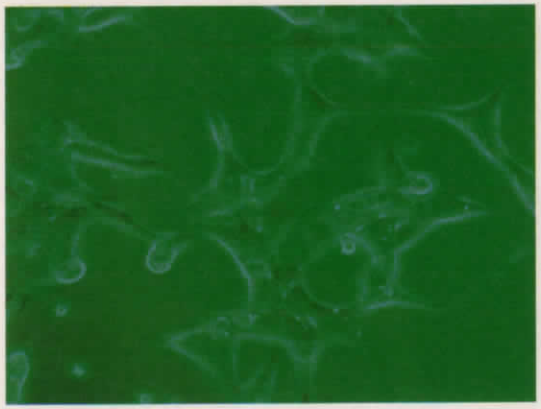


Control

TSA-treated, 12 hours



TSA-treated, 24 hours



TSA-treated, 36 hours

Figure 13

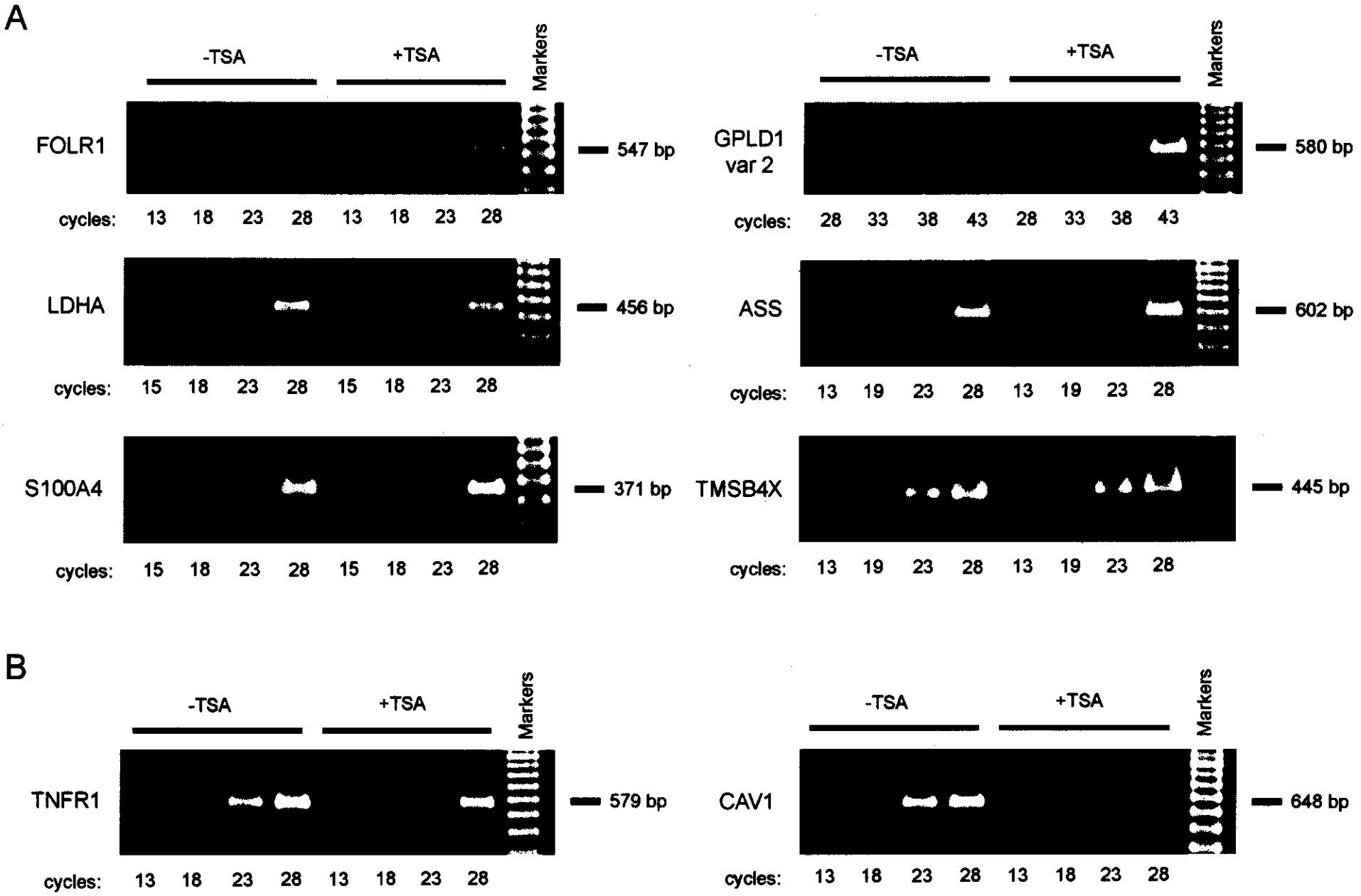

A
-TSA
+TSA
Markers
FOLR1
547 bp
cycles: 13 18 23 28 13 18 23 28
LDHA
456 bp
cycles: 15 18 23 28 15 18 23 28
S100A4
371 bp
cycles: 15 18 23 28 15 18 23 28
GPLD1 var 2
580 bp
cycles: 28 33 38 43 28 33 38 43
ASS
602 bp
cycles: 13 19 23 28 13 19 23 28
TMSB4X
445 bp
cycles: 13 19 23 28 13 19 23 28
B
-TSA
+TSA
Markers
TNFR1
579 bp
cycles: 13 18 23 28 13 18 23 28
CAV1
648 bp
cycles: 13 18 23 28 13 18 23 28

Figure 14

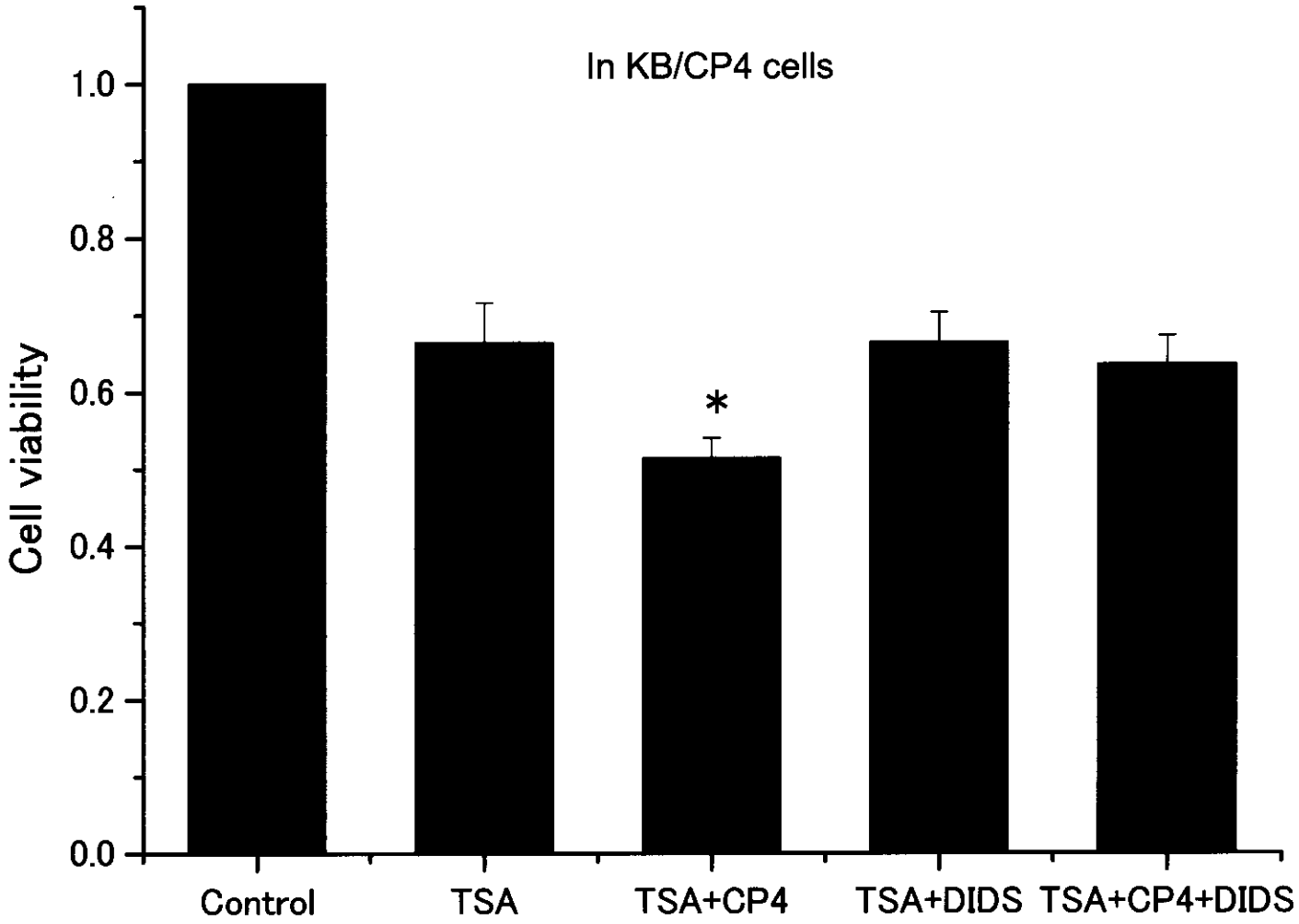
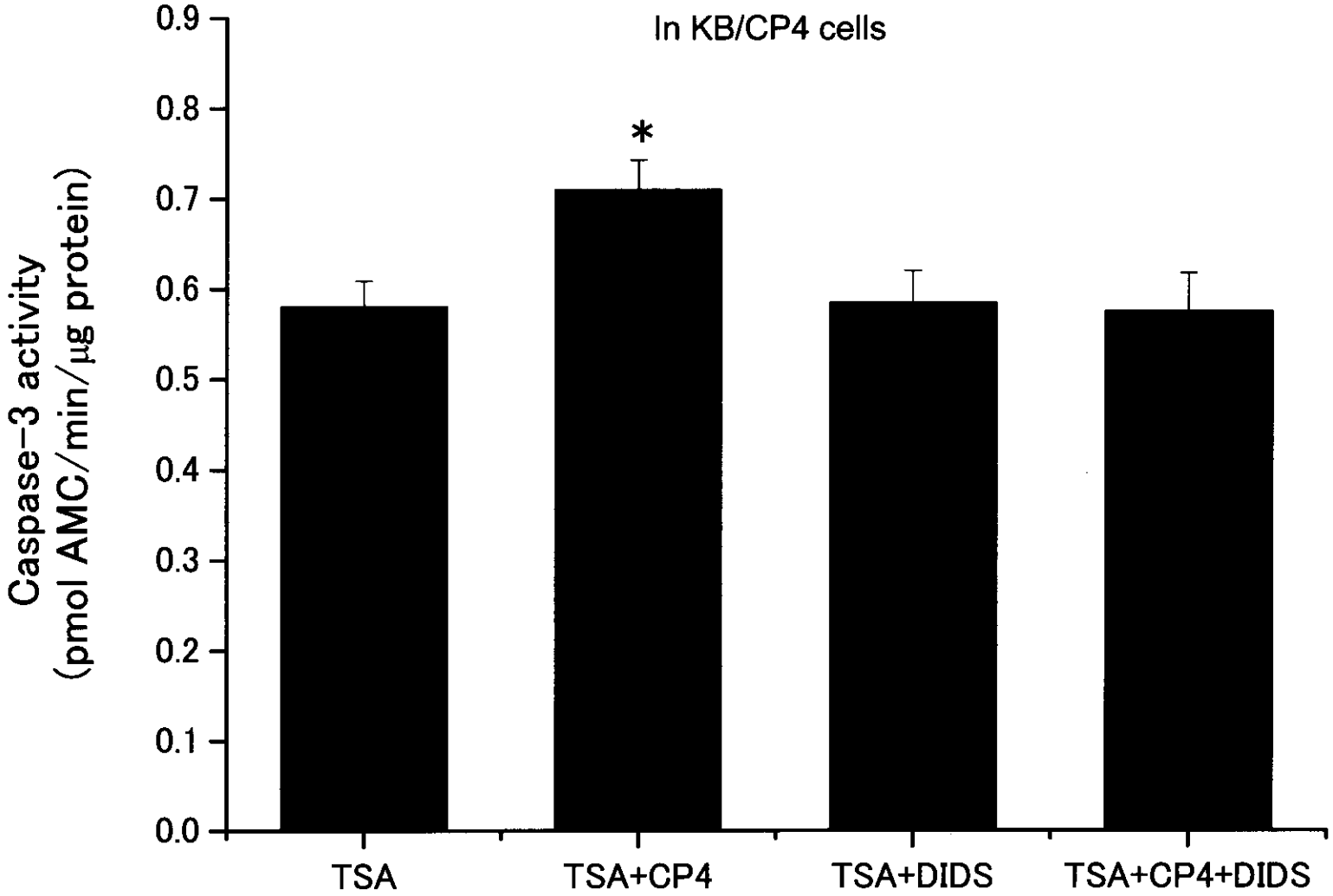

In KB/CP4 cells
Cell viability
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
TSA
TSA+CP4
TSA+DIDS
TSA+CP4+DIDS

Figure 15


In KB/CP4 cells
Caspase-3 activity
(pmol AMC/min/μg protein)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
TSA
TSA+CP4
TSA+DIDS
TSA+CP4+DIDS