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# Protein Kinase C-Independent Correlation Between P-Glycoprotein Expression and Volume Sensitivity of $Cl^-$ Channel

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**Abstract.** The possible correlation between P-glycoprotein (PGP) and volume-sensitive $Cl^-$ channel was examined in a pair of cell lines: a subline of the human epidermoid KB cell (KB-3-1) and the corresponding *MDR1*-transfected cell line (KB-G2). Western blot analysis and indirect immunofluorescence studies indicated that KB-G2, but not KB-3-1, exhibits the PGP expression. Patch-clamp whole-cell recordings showed that osmotic swelling activates $Cl^-$ currents not only in PGP-expressing but also in PGP-lacking cells. The amplitude of the maximal current was indistinguishable between both cells. Activation of protein kinase C (PKC) or loading with a PKC inhibitor failed to affect the swelling-induced activation of the $Cl^-$ currents in both cells. The relation between whole-cell $Cl^-$ currents and cell size measured simultaneously showed that volume sensitivity of the $Cl^-$ channel was augmented by the PGP expression irrespective of the activity of PKC on the plasma membrane. A similar increase in volume sensitivity of the $Cl^-$ channel was also induced by the expression of the ATP hydrolysis-deficient PGP mutant, K433M. We conclude that P-glycoprotein does not represent the volume-sensitive $Cl^-$ channel but that its expression modulates volume sensitivity of the $Cl^-$ channel in a manner independent of its ATPase activity or of the protein kinase C activity.

**Key words:** $Cl^-$ channel — P-glycoprotein — RVD — PKC — Human epidermoid cell

## Introduction

Volume-sensitive outwardly rectifying (VSOR) $Cl^-$ channels have been found in a variety of cell types in association with osmotic cell swelling (Nilius et al., 1994; Strange, Emma & Jackson, 1996) and shown to be actually involved in the regulatory volume decrease (RVD) (Kubo & Okada, 1992; Chan et al., 1994; Gschwentner et al., 1994; Coca-Prados et al., 1996). Although the biophysical and pharmacological properties of the VSOR $Cl^-$ channel have been well characterized (Strange et al., 1996), the molecular identity has not as yet been determined. Based on the experimental data obtained in NIH/3T3 cells transfected with human *MDR1,* it has been hypothesized that P-glycoprotein (PGP), a drug-pumping ABC transporter responsible for multidrug resistance to a broad spectrum of chemotherapeutic agents, switches function to a channel upon osmotic swelling (Valverde et al., 1992; Gill et al., 1992) or that PGP regulates the VSOR $Cl^-$ channel via phosphorylation of PGP by protein kinase C (PKC) (Hardy et al., 1995). Recently, however, we showed that the endogenously expressing PGP molecule is not itself a VSOR $Cl^-$ channel nor a PKC-mediated regulator of the channel in human epithelial cells (Tominaga et al., 1995). In the present study, we then examined whether either of these two hypotheses is applicable for PGP when expressed exogenously in KB cells. The results are totally incompatible with both hypotheses. However, the relation between cell size and the $Cl^-$ current amplitude showed that expression of either PGP or its ATP hydrolysis-deficient mutant does augment the volume sensitivity of the $Cl^-$ channel in a manner independent of PKC activity.

## Materials and Methods

### Cells

The following two sublines of a human mouth epidermoid carcinoma KB cell line were cultured in monolayer using Dulbecco's modified

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Eagle's medium supplemented with 10% fetal calf serum: KB-3-1, which was shown to be highly sensitive to multiple anti-cancer drugs (Akiyama et al., 1985), and KB-G2, which was established by introducing the expression vector for the human *MDR1* gene into KB-3-1 cells (Ueda et al., 1993). KB-G2 was propagated in 100 ng/ml of vinblastine to maintain high expression of PGP.

To obtain KB cells expressing the ATP hydrolysis-deficient PGP mutant, K433M (Gill et al., 1992), in which methionine was substituted for lysine-433 in the first nucleotide-binding domain, KB-3-1 cells were transfected by the MDR1 K433M expression vector with LipofectAMINE (Gibco, Gaithersburg, MD) according to manufacture's direction. Cells were selected in the presence of 0.8 mg/ml geneticin (G418) for 10 days. The expression plasmid for K433M was constructed by fusing the mutated MDR1 cDNA to aminoglycosidase phosphotransferase (neomycin resistant: $neo^r$) gene with an encephalomyocarditis virus 5′ untranslated sequence, known as an internal ribosome entry site (IRES). The IRES fragment was inserted between 3′ end of the MDR1 cDNA and 5′ end of the $neo^r$ gene, and made it possible to coexpress the human MDR1 cDNA using cap-dependent translation initiation and the $neo^r$ gene using cap-independent translation initiation from a bicistronic transcript.

### P-Glycoprotein Analysis

For immunoblotting, membrane proteins were prepared as described previously (Ueda et al., 1992) and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7% gel. Western blot analysis was done with the monoclonal antibody C219 (Centocor, Malvern, PA).

The monolayer cells cultured on glass coverslips were provided for immunofluorescence studies. KB-3-1 and KB-G2 cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 15 min at room temperature. They were washed with PBS, and blocking was made with 3% normal bovine serum in PBS for 30 min. Then they were incubated with 10 μg/ml monoclonal antibody, MRK16 (a gift from T. Tsuruo, University of Tokyo). After wash with PBS they were incubated with biotinylated goat antimouse IgG (Dako, Kyoto, Japan) for 10 min and then incubated with streptavidin fluorescein-5-isothiocyanate (diluted 1:100 with PBS, Dako) for 5 min. After a third wash with PBS, the cells were examined with a fluorescence microscope (Axiovert 100, Zeiss, Jena, Germany; with ×63 objective).

### Protein Kinase C Assay

Total cellular extracts, cytosolic or solubilized membrane fractions were prepared as described (Chambers et al., 1990). PKC activity of the eluants of DEAE-Sephacel columns was determined as described (Kikkawa et al., 1983) except that 75 μM synthetic peptide, Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-OH, and 0.6 mg/ml histone III-S were used as substrates for PKC. Using 7.4 kBq of [$\gamma$-$^{32}$P]ATP each reaction was done at 25°C for 15 min.

### Whole-Cell Patch-Clamp Recordings

After detaching from the plastic substrate the cells were cultured in suspension with agitation for 10 to 90 min. The cells were then placed in a chamber (0.3 ml) and perfused (at a flow rate of 5 ml/min) by gravity feed. A hypotonic challenge was made by switching the perfusate from an isotonic to hypotonic solution (82% osmolality).

Single spherical KB cells were selected for the whole-cell patch-clamp, and $Cl^-$ current recordings were carried out at room temperature (22–26°C), as described previously (Kubo & Okada, 1992). Current activation was monitored under voltage clamp at three different potentials by applying alternating 2-sec pulses (from 0 to ±40 mV). To observe voltage dependence of the current profile especially inactivation kinetics at large depolarizations, 2-sec pulses stepping to ten different voltage levels (from 0 to ±100 mV by 20-mV steps) were alternately applied. The input capacitance of each cell (15–40 pF: mean ~ 28 pF) was measured using the analogue circuit of the patch-clamp amplifier (List EPC-7, Darmstadt, Germany), found to be approximately constant during the experiment even after osmotic swelling, and compensated prior to the current recording.

The isotonic bathing solution contained (in mM): 110 CsCl, 5 $MgSO_4$, 12 HEPES, 8 Tris, and 100 mannitol (pH 7.5; 340 mOsm). A hypotonic bathing solution (280 mOsm) was prepared by reducing the concentration of mannitol from 100 to 40 mM. The low-$Cl^-$ solution was prepared by replacing CsCl with equiosmolar mannitol. The control pipette solution contained (mM) 110 CsCl, 2 $MgSO_4$, 15 Na-HEPES, 10 HEPES, 1 EGTA, 1 $Na_2$-ATP, and 50 mannitol (pH 7.45); and the osmolarity was set at 300 mOsm to prevent spontaneous cell swelling under the whole-cell configuration, presumably due to poorly diffusible osmolytes within the cytosol (Kubo & Okada, 1992). A hypertonic pipette solution (360 mOsm) which was prepared by increasing the mannitol concentration from 50 to 110 mM was used in some experiments to facilitate osmotic swelling.

### Cell Size Measurements

During whole-cell current recordings in KB cells dialyzed with the hypertonic pipette solution, cell size (mean diameter ~ 18 μm) was simultaneously measured by time-lapse videomicroscopy in the isotonic or hypotonic bathing solution. Cell images were recorded with a CCD camera (Ikegami, ICD-42AC, Tokyo) and a videocassette recorder. Two-dimensional cell size was evaluated by the square of diameter of the spherical cell. When osmotic swelling was associated with the formation of membrane blebs, the data were discarded. However, the data were accepted after fusion of a number of blebs gave rise to rounding the cell. The relative surface area was calculated by normalizing the cell size under the whole-cell mode against the cell size just before attaining the whole-cell mode.

### Chemicals

All the agents except for EGTA (Nacalai Tesque, Kyoto), 12-O-tetradecanoyl-phorbol 13-acetate (TPA; Wako, Osaka), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7; Biochem. Prod. Life Sci., Tokyo), [$\gamma$-$^{32}$P]ATP and a synthetic peptide Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-OH (Amersham, Buckinghamshire, England) were from Sigma (St. Louis, MO). Immediately before use, TPA (30 nM) or 4-acetamido-4′-isothiocyanostilbene (SITS; 10 μM) was added to the bathing, and H-7 (10 μM) was to the pipette solution. The vehicle (ethanol for TPA or dimethyl sulfoxide for H-7 and SITS) alone at the employed concentrations (0.1% or less) did not affect the VSOR $Cl^-$ current.

### Statistical Analysis

Statistical differences of the data were evaluated by Student's $t$ test and considered significant at $P < 0.05$. Data are presented as mean ± SE.

## Results

### Expression of P-Glycoprotein is Distinctly Different Between KB-3-1 and KB-G2 Cells

Figure 1 shows Western blot analysis with a PGP-specific monoclonal antibody, C219. The results

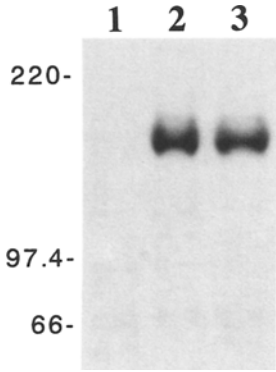


**Fig. 1.** Western blot analysis for P-glycoprotein expression using the monoclonal antibody, C219. Immunoblot of membrane protein fraction (20 mg) from untransfected KB-3-1 (lane 1), KB/K433M (lane 2) and KB-G2 (lane 3) in a 7% SDS-PAGE. Molecular size standards are indicated in kDa at the left.

showed that membrane fractions of KB-G2 (lane 3), but not parental KB-3-1 (lane 1), cells express PGP, as reported previously (Ueda et al., 1993). The KB cells transfected with cDNA of the K433M mutant were also found to exhibit the protein expression in the membrane fraction (lane 2). Since both the ATP hydrolysis-deficient mutant and the wild type PGP were detected as 170 kDa corresponding to the fully glycosylated form, it appears that the normal processing to the plasma membrane took place not only for the wild but also for the mutant PGP.

To directly visualize the difference in PGP expression between KB-G2 and KB-3-1 cells particularly on the plasma membrane, indirect immunofluorescence studies were performed using another PGP-specific monoclonal antibody, MRK16. KB-G2 cells showed distinct PGP expression particularly in the plasma membrane region, whereas KB-3-1 cells virtually lacked PGP ($n$ = 10, *data not shown*).

### Activity of Volume-Sensitive $Cl^-$ Channels in PGP-Expressing and -Lacking KB Cells is Indistinguishable

As shown in Fig. 2*A*, not only in PGP-expressing KB-G2 but also PGP-lacking KB-3-1 cells were found to respond to hypotonic challenge (82% osmolality) with activation of outward and inward currents at positive and negative potentials, respectively, under whole-cell voltage-clamp. Maximal activation of the current was observed in association with full swelling. When the extracellular $Cl^-$ concentration was reduced from 110 to 40 mM, the zero-current (reversal) potential shifted by around +20 mV in both cell lines ($n$ = 4, *data not shown*), indicating that the volume-activated current was mainly carried by $Cl^-$ ions. The currents were markedly suppressed by a stilbene-derivative $Cl^-$ channel blocker, SITS (10 μM, $n$ = 3, *data not shown*). The swelling-activated $Cl^-$ currents always exhibited outward rectification and time-dependent inactivation at large positive potentials (Fig. 2*A*, Insets).

The magnitude of volume-sensitive outwardly rectifying (VSOR) $Cl^-$ currents was indistinguishable between KB-3-1 and KB-G2 cells after attaining the steady state of full swelling. The mean values of maximal current density recorded at +40 mV were 103 ± 7 pA/pF ($n$ = 27) in KB-3-1 cells and 113 ± 6 pA/pF ($n$ = 33) in KB-G2 cells ($P$ = 0.289).

### Effects of an Activator and an Inhibitor of Protein Kinase C on Volume-Sensitive $Cl^-$ Currents in PGP-Expressing and -Lacking KB Cells are Indistinguishable

Recently, it has been reported that prior treatment with a PKC activator, TPA (30 nM), prevents induction of $Cl^-$ currents by osmotic swelling in PGP-overexpressing but not PGP-lacking NIH/3T3 cells (Hardy et al., 1995). As shown in Fig. 2*B*, however, prior treatment with TPA (30 nM for 5 to 15 min) failed to prevent swelling-induced activation of $Cl^-$ currents not only in KB-3-1 but also in KB-G2 cells. The same results were obtained with 200 nM TPA (*not shown*). After pretreatment with 30 or 200 nM TPA, the mean maximal $Cl^-$ current densities recorded at +40 mV were 101 ± 8 pA/pF ($n$ = 13) and 114 ± 8 pA/pF ($n$ = 11) in fully swollen KB-3-1 and KB-G2 cells, respectively. These values are not significantly different from each other ($P$ = 0.273) and from those in both cell lines without TPA treatment ($P$ = 0.902 and 0.922). Fully pre-activated VSOR $Cl^-$ currents were also unaffected by bath application of TPA (30 nM) in both cells (Fig. 2*C*). The mean maximal current densities at +40 mV were 97 ± 8 ($n$ = 8) and 103 ± 9 pA/pF ($n$ = 8; $P$ = 0.623) in KB-3-1 cells, and 107 ± 9 ($n$ = 6) and 115 ± 7 pA/pF ($n$ = 6; $P$ = 0.492) in KB-G2 cells, before and after bath application of TPA, respectively.

KB-3-1 cells were already shown to exhibit endogenous PKC activity which could be stimulated by TPA (Chambers et al., 1990). Similarly, KB-G2 cells were found to have endogenous PKC activity and to respond to the PKC activator (30 or 200 nM TPA for 10 min) with translocation of PKC from the cytosol to the membrane, as summarized in the Table.

As shown in Fig. 2*D*, loading the cells with H-7 (10 μM for over 10 min), which is known to inhibit PKC activity in KB cells (Chambers et al., 1990), could not significantly affect activation of VSOR $Cl^-$ currents in either KB-3-1 or KB-G2 cells. The mean maximal current densities at +40 mV were 129 ± 11 pA/pF ($n$ = 5) in KB-3-1 and 128 ± 22 pA/pF ($n$ = 7) in KB-G2 in the presence of H-7, and were not significantly different

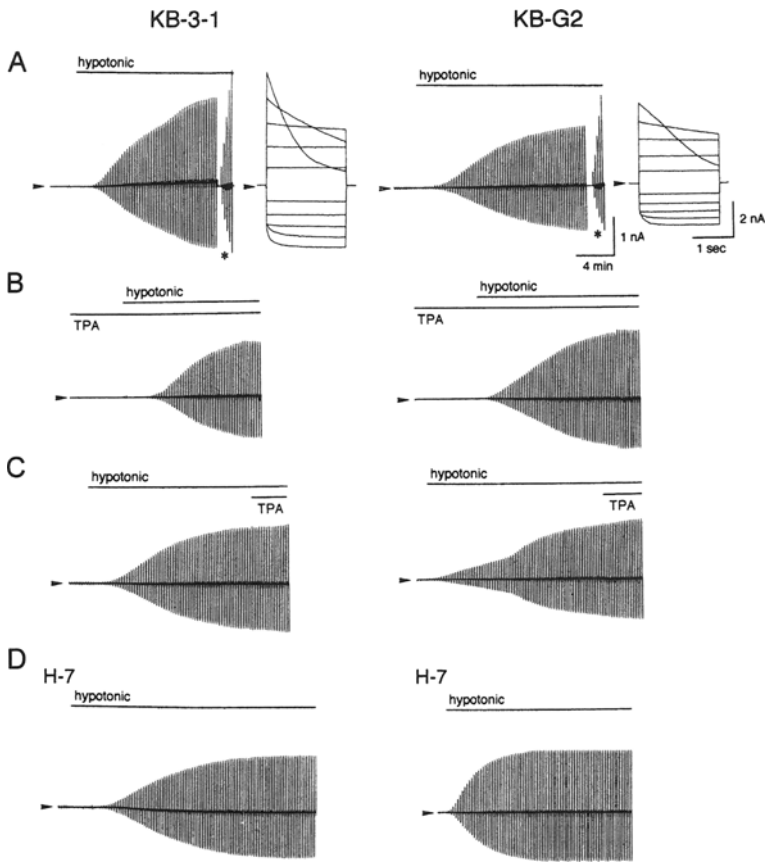


**Fig. 2.** Volume-sensitive $Cl^-$ currents recorded in KB-3-1 and KB-G2 cells. Representative traces of whole-cell $Cl^-$ current responses to hypotonic challenge recorded during application of alternating 2-sec pulses from 0 to ±40 mV in KB-3-1 (left) and KB-G2 cells (right) dialyzed with the control pipette solution. Arrowheads indicate the zero level, and horizontal lines above the current traces indicate the time of hypotonic challenge or application of TPA. Neither TPA nor H-7 affected cell swelling induced by hypotonic challenge. (*A*) Swelling-induced $Cl^-$ currents under control conditions. At asterisks step pulses were applied from 0 to ±100 mV in 20-mV increments after changing the gain of chart recorder by half. *Insets:* Expanded traces of the current responses recorded at asterisks. (*B*) Swelling-induced $Cl^-$ currents in the presence of TPA (30 nM) added 5 min before and during hypotonic challenge. (*C*) Swelling-induced $Cl^-$ currents in the absence and presence of TPA (30 nM) added after attaining full swelling. (*D*) Swelling-induced $Cl^-$ currents in the presence of H-7 (10 μM) added over 10 min before and during hypotonic challenge.

**Table.** PKC activity and its distribution in KB-3-1 and KB-G2 cells

| Cell line | PKC activity[a] | PKC distribution[b] (% cytosol/membrane) | | |
|---|---|---|---|---|
| | | −TPA | +TPA (30 nM) | +TPA (200 nM) |
| KB-3-1 | 126 ± 28 (n = 4) | 70/30[c] | — | 17/83[c] |
| KB-G2 | 134 ± 32 (n = 4) | 92/8 | 56/44 | 17/83 |

[a] Expressed as pmol phosphate transferred to a synthetic peptide and histone III-S/min/$10^7$ cells.
[b] Data shown are from a representative experiment where cells were either unstimulated (−TPA) or stimulated with 30 or 200 nM TPA for 10 min (+TPA).
[c] Data from Chambers et al. (1990).

from each other ($P = 0.966$) and from the control values in the absence of H-7 ($P = 0.162$ and 0.533).

### PGP-Expressing KB Cells Exhibit Higher Sensitivity of the $Cl^-$ Channel to Volume Expansion than PGP-Lacking KB Cells

Volume sensitivity was assessed by plotting the VSOR $Cl^-$ current density against the relative surface area measured simultaneously. As shown in Fig. 3*A* (open circles), KB-3-1 cells showed a nearly linear relationship between the current density and the relative surface area in a certain range above the threshold (at the relative surface area of 1.2–1.3). This relation was not largely affected by the presence of TPA (200 nM) added before and after the hypotonic challenge (Fig. 3*A:* open triangles).

As shown in Fig. 3*A* (solid circles), KB-G2 cells exhibited a steeper slope of the current density increase in response to increases in the relative surface area (especially in the initial region up to around 1.8–2.0). Increased volume sensitivity in the PGP-expressing cells was not essentially affected by the presence of TPA (200 nM) added throughout the experiments (Fig. 3*A:* solid triangles). When the K433M mutant of PGP was expressed, the volume sensitivity of VSOR $Cl^-$ channel was again found to be augmented, as shown in Fig. 3*B* (solid squares).

## Discussion

The MDR1 gene product, P-glycoprotein (PGP), is a member of the ABC (ATP-binding cassette) superfamily and acts as an ATP-dependent, active transporter expel-

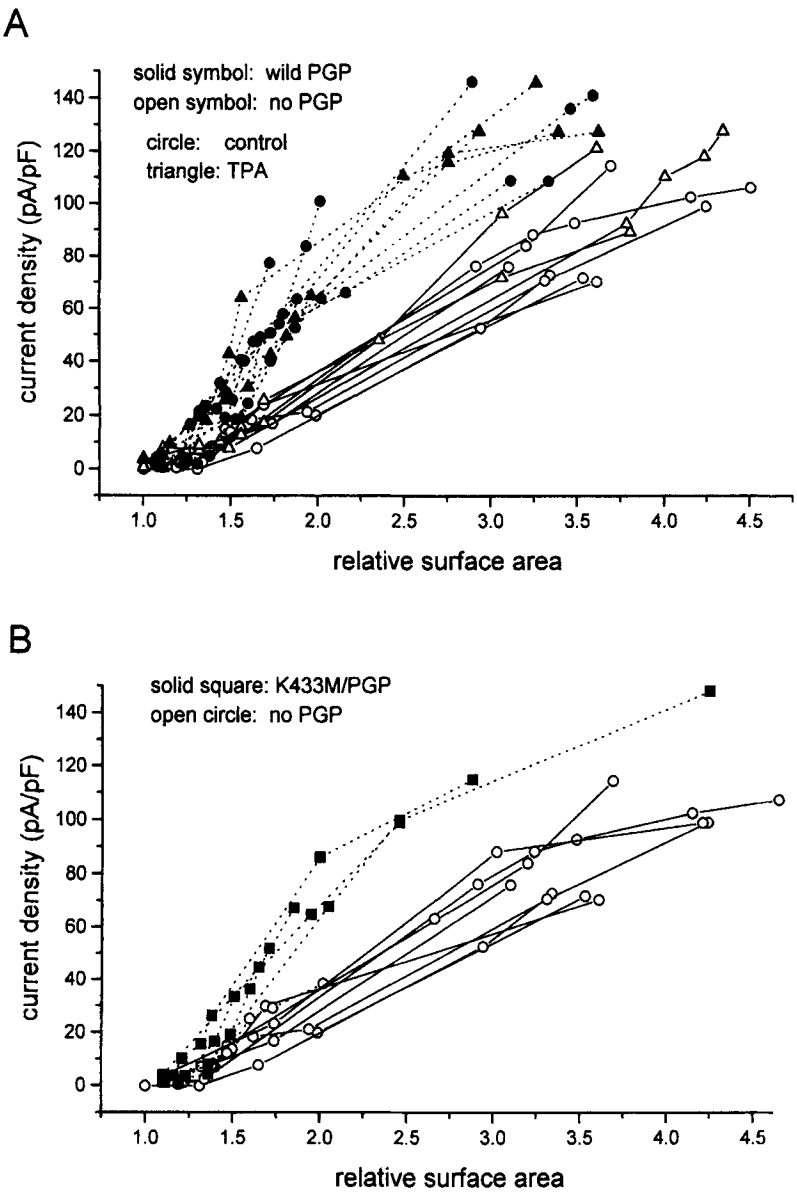


**Fig. 3.** Volume-sensitivity of the $Cl^-$ currents in KB-3-1 and KG-G2 cells. Relationship between the VSOR $Cl^-$ current density and the relative surface area monitored simultaneously in KB cells dialyzed with the hypertonic pipette solution. The perfusate was, at first, the isotonic bathing solution, and then switched to the hypotonic bathing solution. (*A*) Increased volume sensitivity of $Cl^-$ currents in KB-G2 cells expressing wild PGP (solid symbols) compared to those in PGP-lacking KB-3-1 cells (open symbols) in the absence (circles) or presence (triangles) of 200 nM TPA added 5–10 min before and during whole-cell recordings. The slope estimated by regression analysis was 36.5 ± 1.2 for all the points of KB-3-1 ($n$ = 55) or 52.7 ± 3.3 for those of KB-G2 ($n$ = 68; $P < 0.05$). The slope without TPA was not significantly different from that with TPA. (*B*) Increased volume sensitivity of $Cl^-$ currents in KB cells expressing the K433M mutant of PGP (solid squares) compared to those in PGP-lacking KB-3-1 cells (open circles). The slopes estimated by regression analysis were 34.3 ± 1.4 and 55.9 ± 4.0 ($P < 0.05$) for all the points of PGP-lacking KB-3-1 ($n$ = 43) and of K433M/PGP-expressing KB-3-1 ($n$ = 25), respectively.

ling drugs from the cytosol of multidrug-resistant cells (Higgins, 1992). Two intriguing hypotheses have been proposed by Higgins and his collaborators (Valverde et al., 1992; Gill et al., 1992; Hardy et al., 1995) concerning the relation between PGP and swelling-induced $Cl^-$ conductances.

At first, PGP was suggested to function as the volume-sensitive $Cl^-$ channel itself upon osmotic swelling (Valverde et al., 1992; Gill et al., 1992), mainly based on the observation that transfection with the *MDR1* gene resulted in the expression of both PGP and VSOR $Cl^-$ currents. In the present study no correlation was found between the level of PGP expression (Fig. 1) and the magnitude of the maximal VSOR $Cl^-$ current (Fig. 2*A*). Therefore, it is clear that PGP is not itself the molecular identity of volume-sensitive $Cl^-$ channel in the human epidermoid cells. This conclusion is completely consistent with an increasing number of recent reports of lack of correlation between PGP expression and swelling-activated $Cl^-$ conductance in many cell species (Altenberg et al., 1994; Dong et al., 1994; Ehring, Osipchuk & Cahalan, 1994; Rasola et al., 1994; De Greef et al., 1995; Viana et al., 1995; also *see* Borst et al., 1993) and also with our recent observation that antisense-induced abolition of endogenous PGP expression failed to affect the magnitude of maximal VSOR $Cl^-$ current in a human epithelial cell line (Tominaga et al., 1995).

Simultaneous measurements of cell size and whole-cell $Cl^-$ currents in the present study, however, clearly demonstrated that the VSOR $Cl^-$ channel in PGP-expressing KB cells exhibits higher sensitivity to cell swelling than in PGP-lacking KB cells (Fig. 3*A*). Like-

wise, increased volume sensitivity in association with PGP expression was reported based on the observation that swelling-activated $Cl^-$ conductance was induced by a lesser hypotonicity after PGP-overexpressing in NIH/3T3 cells (Luckie et al., 1994) and LR73 cells (Valverde et al., 1996).

Volume sensitivity of the $Cl^-$ channel was also augmented by the expression with the K433M mutant of PGP (Fig. 3*B*) which was shown to exhibit no ATPase activity (Loo & Clarke, 1995 but reported to induce the volume-activated $Cl^-$ channel activity in S1 cells (Gill et al., 1992). Therefore, the ATPase or pump activity of PGP is not indispensable for the modulatory PGP action on the $Cl^-$ channel.

PKC, a $Ca^{2+}$- and phospholipid-dependent protein kinase, is implicated in the regulation of a variety of cell functions (Nishizuka, 1984; 1986). The pump function of PGP was also reported to be modulated by its phosphorylation due to PKC activation in KB cells (Chambers et al., 1990) and other cell species (Posada, Vichi & Tritton, 1989; Ma et al., 1991). Recently, PKC-mediated phosphorylation of PGP has been proposed to regulate the volume-sensitive $Cl^-$ channel activity on the basis of the observation that pretreatment with a PKC activator, TPA, prevented swelling-induced activation of the $Cl^-$ current in NIH/3T3 cells transfected with human *MDR1* but not the control parental cells lacking detectable PGP expression (Hardy et al., 1995). This PKC-mediated regulator hypothesis is, however, at variance with the present study, because no effect of TPA (Fig. 2*B, C*) or H-7 (Fig. 2*D*) on the magnitude of maximal VSOR $Cl^-$ currents was observed in both PGP-lacking and -expressing KB cells, and because osmotic induction of $Cl^-$ currents was not suppressed by facilitated (Fig. 3*A*) in PGP-expressing KB cells in spite of the detectable basal activity of membrane-associated PKC (Table). Furthermore, volume sensitivity of the $Cl^-$ channel was not largely affected by TPA irrespective of the PGP expression level in KB cells (Fig. 3*A*), although TPA actually activated PKC leading to its translocation to the plasma membrane (Table). In addition, volume sensitivity of the $Cl^-$ channel in KB-G2 cells was not affected by loading with H-7 (10 μM: Y. Okada, *unpublished observations*). Therefore, it appears that the PKC activity does not mediate the modulating action of PGP on the VSOR $Cl^-$ channel.

Taken together, it is concluded that P-glycoprotein is not responsible for the molecular identity of the volume-sensitive $Cl^-$ channel but modulates volume sensitivity of the channel in a manner independent of its ATPase activity or of the protein kinase C activity. The mechanistic aspect of the interaction between P-glycoprotein and the volume-sensitive $Cl^-$ channel is subject to further studies.

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