Involvement of PKC-α in regulatory volume decrease responses and activation of volume-sensitive chloride channels in human cervical cancer HT-3 cells

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(Received 9 March 1998; accepted after revision 29 June 1998)

1. The present study was carried out to identify the specific protein kinase C (PKC) isoform involved in regulatory volume decrease (RVD) responses, and to investigate the signal transduction pathways underlying the activation of volume-sensitive chloride channels in human cervical cancer HT-3 cells. The role of Ca^{2+} in RVD and in the activation of chloride currents was also studied.

2. The time course of RVDs was prolonged by microinjection of PKC-α antibody but not by PKC-β or PKC-γ antibody, and also by exposure to Ca^{2+}-free medium, in particular when combined with microinjection of EDTA. Immunofluorescence staining showed that hypotonic superfusion evoked the translocation of PKC-α to the cell membrane, whereas PKC-β or PKC-γ remained unaffected. The translocation of PKC-α was observed a few minutes after hypotonic stress, reaching peak intensity at 30 min, and returned to the cytoplasm 60 min after hypotonic exposure. Western blot analyses showed an increased PKC-α level in terms of intensity and phosphorylation in the cell membrane, while neither PKC-β nor PKC-γ was activated upon hyposmotic challenge.

3. Whole-cell patch-clamp studies demonstrated that neomycin and PKC blockers such as staurosporine and H7 inhibited volume-sensitive chloride currents. The inhibitory effect of neomycin on chloride currents can be reversed by the PKC activator phorbol 12-myristate, 13-acetate (PMA). Moreover, the PKC inhibitor and PKC-α antibody, but not PKC-β or PKC-γ antibody, significantly attenuated the chloride currents. The activation of volume-sensitive chloride currents were insensitive to the changes of intracellular Ca^{2+} but required the presence of extracellular Ca^{2+}.

4. Our results suggest the involvement of PKC-α and extracellular Ca^{2+} in RVD responses and the activation of volume-sensitive chloride channels in HT-3 cells.

The maintenance of homeostasis is a fundamental property of cells, and all cells possess mechanisms to regulate their volume precisely during mitosis and osmotic challenge. Cell volume homeostasis does not simply mean volume constancy, but rather serves as the integration of events in regulating cell function (Haussinger, 1996; Lang *et al.* 1998). The process of volume adaptation observed after a hypotonic stress is the regulatory volume decrease (RVD) responses which may be accomplished by separate activation of conductive K^+ and Cl^- pathways (Hoffmann & Simonsen, 1989; Hoffmann & Dunham, 1995). We have previously demonstrated that volume-sensitive chloride currents, leading to RVD and taurine transport, were significantly activated only in carcinomas *in situ* and invasive cervical cancer, but not in non-cancerous cells such as human papillomavirus (HPV)-immortalized cell lines and normal cervical cells (Chou *et al.* 1995; Shen *et al.* 1996; Chou *et al.* 1997). Our data suggest that the activation of these channels is associated with the process involved in the malignant transformation of human cervical epithelium. Chloride currents activated by cell swelling have been reported in a number of epithelial and non-epithelial cells. However, these currents vary substantially in biophysical properties (Strange *et al.* 1996; Okada, 1997). In addition, the signal transduction pathways that regulate these currents are not clear.

By using a pharmacological approach we recently demonstrated that a protein kinase C (PKC) isoform regulated by upstream phospholipase C (PLC) is involved in the RVD responses of cervical cancer cells, whereas PKC isoforms unrelated to PLC signalling are implicated in the RVD of the HPV-immortalized and normal cervical

epithelia (Shen *et al.* 1998). In cervical cancer cells such as HT-3 and CasKi cells, the phorbol ester phorbol 12-myristate, 13-acetate (PMA) facilitates, while the PLC inhibitor neomycin and PKC inhibitors such as staurosporine and H7 dihydrochloride (H7) inhibit the RVD responses. Further experiments have shown that the action of neomycin is upstream of PKC, suggesting the involvement of conventional PKCs. The PKC family is a heterogeneous family of phospholipid-dependent kinases that can be divided into three categories on the basis of cofactor requirements and structure (Hug & Sarre, 1993; Nishizuka, 1995; Jaken, 1996). Conventional PKCs require both calcium and diacylglycerol (DAG) or phorbol ester as cofactors. Activated receptors of tyrosine kinase and G protein-coupled classes are able to recruit conventional PKC for intracellular signalling. These receptor-induced signals initiate the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) by PLC to inositol 1,4,5-trisphosphate (IP_3) and DAG, which lead to increased intracellular calcium concentration and PKC activation. Novel PKCs require only DAG or phorbol ester, whereas atypical PKCs do not require calcium or DAG for maximal activity. PMA and conventional PKC blockers have been shown to have varying effects on RVD or volume-sensitive chloride currents in different cell types. However, little is known about the specific PKC isoform involved in these responses. The present study was conducted to identify the specific PKC isoform involved in RVD responses, and to use the patch-clamp technique to assess the signal transduction pathways involved in the regulation of volume-sensitive chloride channels in human cervical cancer HT-3 cells.

METHODS

Cell culture

The cervical cancer cell line HT-3 was obtained from the American Type Culture Collection (Rockville, MD, USA). The growth medium for HT-3 cells was Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum (Gibco), 80 IU ml^{-1} penicillin and 80 μg ml^{-1} streptomycin (Sigma). HT-3 cells were maintained at 37 °C in a CO_2–O_2 (5%–95%) atmosphere and used 2–3 days after subculturing.

Measurements of cell volume

Cell volume was measured as described previously (Shen *et al.* 1998). Briefly, cells were grown in tissue culture flasks and were harvested by trypsinization. Cells (3×10^6) were suspended in 5 ml of an isotonic solution for 10 min. Then, 500 μl aliquots were transferred and allowed to stand for approximately 20 min in Petri dishs to achieve cell attachment. A 2 ml bath, which was continuously superfused at a rate of 2 ml min^{-1} with isotonic solution or hypotonic solution (210 mosmol l^{-1}) with or without the chemical tested at room temperature (22–25 °C), was then applied. In order to monitor the change of cell size, a microscope was coupled to a video camera system with magnification up to ×400 and continuously stored on a video cassette recorder (National Inc., Tokyo, Japan). The majority of cells observed were spheroid. Therefore, the cell diameter was defined as the mean of the longest axis plus the shortest axis. Data were presented as a percentage of starting volume (V/V_0), as a function of time.

Microinjection of cells

Blockers that were poorly permeant through the cell membrane were delivered into the cytoplasm by microinjection. These included EGTA, and antibodies for conventional PKCs (α, β and γ isoforms). This technique is reported to be efficient for the transfer of macromolecules into cultured cells (Graesmann & Graesmann, 1983; Ridley, 1995). To ensure that the procedure of microinjection caused no damaging effect, the RVD responses of at least twenty HT-3 cells injected with pipette solution were analysed. The results showed that the RVD responses were not significantly affected by microinjection. For preparing the micropipettes for microinjection, GD-1 glass capillaries (Narishige Scientific Instrument Lab., Tokyo, Japan) were heated and pulled by gravity using a two-step, vertical micropipette puller (PC-10; Narishige). A coarse positioning manipulator (ONM-1; Narishige) and ergonomic joystick micromanipulator (ONO-125; Narishige) were used to position the micropipette near the cell. Cells were injected with 0·1 pl of chemicals by microinjector (IM-6, Narishige). The composition of the pipette solution was as follows (mM): KCl 130·0, $MgCl_2$ 2·0 and Hepes 5·0 (buffered by NaOH to pH 7·2).

Immunofluorescence study

To observe the intracellular localization of PKC isozymes, immunofluorescence staining was performed as described by Franchi-Gazzola *et al.* (1996) with the following modifications. Cells were seeded in two-well LabTech Chamber Slides (Nunclon, Naperville, IL, USA) at a concentration of 2×10^4 cells $well^{-1}$ in DMEM supplemented with 10% fetal calf serum. After the hypotonic superfusion, cells were fixed with 3·7% formaldehyde for 10 min at −20 °C, washed with PBS, and incubated with the PKC isoform-specific antibodies (Transduction Laboratories, Lexington, KY, USA) at a concentration of 2 μg ml^{-1} for 45 min at 37 °C. The slides were washed three times with PBS, and then incubated at 37 °C with fluorescein-conjugated anti-mouse IgG antibody (Transduction Laboratories) at a concentration of 20 μg ml^{-1} for 35 min. Cells were examined with a Nikon microscope equipped for epifluorescence with appropriate filters for detecting fluorescence. Photomicrographs were taken on Kodak ISO 400 film.

Preparation of subcellular fractions and Western blot analysis

Preparation of subcellular fractions for Western blotting was performed as described by Chang *et al.* (1992) with minor modifications. Cervical cells in 15 cm Petri dishes were washed with PBS and scraped with a Teflon sheet into 1 ml of lysis buffer (50 mM Tris-HCl (pH 7·4), 0·5 mM EGTA, and 1 mM phenylmethylsulphonyl fluoride). Cells were then sonicated in a Heat-Systems-Ultrasonics Model W-375 sonicator. The homogenate was centrifuged at 10 000 *g* for 20 min at 4 °C in a Sorvall SM 24-9 ultracentrifuge. The resulting supernatant was recentrifuged at 147 000 *g* for 1 h at 4 °C in a Beckman L7-65 ultracentrifuge. The resulting pellet was resuspended in 1 ml of lysis buffer containing 0·25% Tween 20 (Merck), allowed to stand on ice with occasional stirring for 30 min to dissolve the lipid layer, and then centrifuged again at 147 000 *g* for 1 h at 4 °C. The pellet was suspended in 1 ml of 50 mM Tris-HCl, pH 7·4, unless stated otherwise, and designated as the particulate (membrane) fraction. The supernatant was designated as the cytosolic fraction. Protein concentration was determined with the Bio-Rad protein assay.

Fifteen micrograms of protein from both the cytosolic and particulate fractions was denatured in SDS lysis buffer and loaded into duplicate 10% SDS–polyacrylamide gels. After electrophoresis at 100 V for 3 h in SDS–PAGE running buffer (25 mM Tris,

250 mM glycerine (pH 8·3) and 0·1% SDS), one of the gels was stained with colorimetric Coomassie Brilliant Blue (Sigma) as a quantitative control, and the other was transferred to the flurotrans polyvinylidine difluoride membrane (Stratagene, La Jolla, CA, USA) and blocked with 5% skimmed milk (Difco) in phosphate-buffered saline–Tween (PBS-T; 100 mM NaCl, 80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 0·2% Tween-20; pH 7·5) solution at 4 °C overnight. After washing with PBST and PBS, the membrane was hybridized with an isoform-specific PKC antibody at 37 °C for 1 h. The membrane was then washed and hybridized with the monoclonal anti-mouse IgG conjugated with horse peroxidase (Oncogene Science, Uniondale, NY, USA) at 25 °C for 1 h. The membrane was then exposed to the X-ray film (Fuji, Osaka, Japan) for 10 min after the ECL detection reagent (Amersham) was added (Kashani-Sabet *et al.* 1992). Positive control for each PKC isoform was included in the blottings. The intensity of each band appearing in the Western blot was then analysed by a laser densitometer (PD-120, Molecular Dynamics; Sunnyvale, CA, USA).

Chemicals and solution

Erbstatin analogue, staurosporine, H7, PMA, 3-isobutyl-1-methylxanthine (IBMX), and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) were purchased from Research Biochemicals. Isoform-specific conventional PKC antibodies (α, β and γ) and the fluorescein-conjugated anti-mouse IgG antibody were purchased from Transduction Laboratories, whereas PKC inhibitor, which corresponds to the PKC pseudosubstrate site that maintains the PKC inactive state by occupying the PKC active site, was from Santa Cruz (SC-3007; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The other chemicals were obtained from Sigma unless stated otherwise. The osmolarity of the solution was measured using a vapour pressure osmometer (Wescor 5500, Schlag Meßinstrumente, Gladbach, Germany). The isotonic solution (290 ± 5 mosmol l^{-1}) was modified lactated Ringer solution which contained (mM): Na^+, 140; Cl^-, 135; K^+, 3·5; Ca^{2+}, 1·5; and lactate, 10; titrated to pH 7·4 with NaOH. The hypotonic solution (210 ± 5 mosmol l^{-1}) was adjusted with 1 mosmol l^{-1} H_2O to the

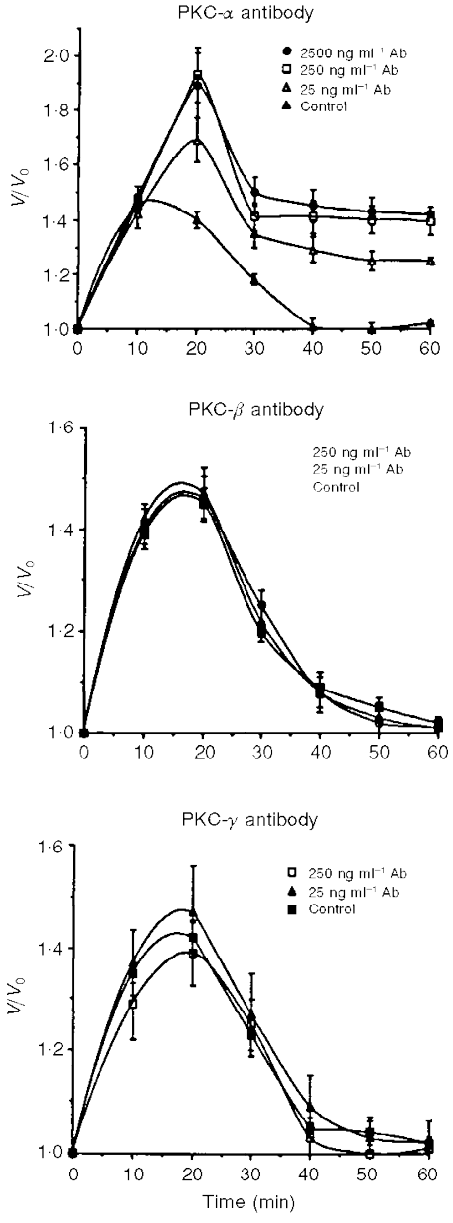

Figure 1. Effects of isoform-specific PKC antibodies on the time course of RVD in HT-3 cells

Examples of time course of volume changes in HT-3 cells following superfusion with 210 mosmol l^{-1} hypotonic bath solution are shown. The isoform-specific PKC antibody was delivered by microinjection. Ten minutes after microinjection, hypotonic solution was perfused. The y-axis (V/V_0) depicts the cell volume at the indicated times divided by the cell volume at zero time. Each point represents mean ± S.E.M. ($n = 15$ cells) from three separate experiments.

desired values. Nominally Ca^{2+}-free medium was prepared by omitting addition of calcium. To measure the resting membrane potential, the pipette solution contained (mM): KCl, 135; EGTA, 0·1; $MgCl_2$, 2; ATP, 3; GTP, 0·1; and Hepes, 12; pH 7·2. In experiments for recording hypotonicity-induced Cl^- current, KCl inside the pipette solution was replaced with equimolar CsCl, and pH was adjusted to 7·2 with CsOH.

Electrophysiological measurements

Whole-cell membrane currents were measured using ruptured patches as described previously (Chou *et al.* 1995; Shen *et al.* 1996). Cells were bathed at room temperature and continuously superfused at a rate of 2 ml ml^{-1} with isotonic or hypotonic solution. Patch pipettes for whole-cell recording were heated and pulled from borosilicate glass capillaries (Intracel Ltd, Cambridge, UK) by gravity using a two-step, vertical micropipette puller (PC-10; Narishige). When the pipettes were connected to the input stage of an Axonpatch-200A amplifier (Axon Instruments), their DC resistance varied between 3 and 5 MΩ. A three-dimensional micromanipulator (Model 860MC3, Newport Co., Irvine, CA, USA) was used to position the pipette near the cell. The liquid junction potential was corrected immediately before the pipette was attached to cells. In order to validate the experimental conditions, when the whole-cell-recording mode was established, some cells were allowed to remain stabilized for about 5–10 min. In experiments designed to construct the current *versus* voltage (I–V) relationships, either square command pulses with a duration of 100 ms from the holding potential to various potentials or ramp command pulse with a duration of 500 ms for −80 to +40 mV were employed.

Data recording and analysis

Data were digitized and analysed using pCLAMP software (version 6.0.3, Axon Instruments). All values were reported as the mean ± S.E.M. (standard error of the mean). ANOVA and Student's paired or unpaired t tests were used for statistical analyses. Differences between values were considered significant when $P < 0{\cdot}05$.

RESULTS

Volume response to hypotonic stress

In HT-3 cells, the typical RVD responses to 210 mosmol l^{-1} hypotonic solution were an initial and rapid osmotic swelling to peak volume at 10–20 min, then a rapid shrinkage in the following 10–20 min, and finally a more gradual decrease to the original cell size at 40–60 min (Fig. 1, control group). To characterize which PKC isoform is involved in the regulation of RVD, three different types of monoclonal antibodies (anti-α, anti-β, anti-γ) were microinjected into HT-3 cells 10 min before hypotonic challenge. As shown in

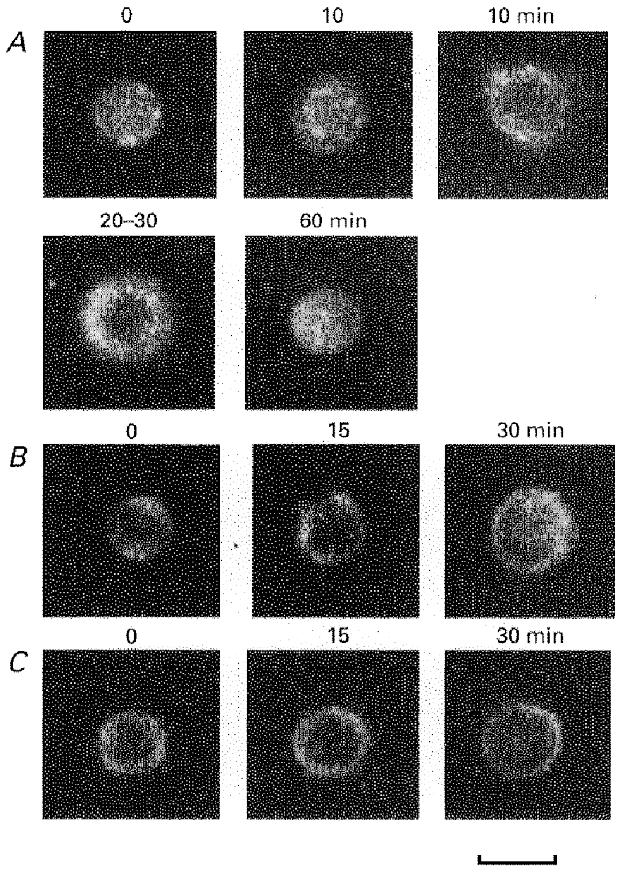

Figure 2. Immunofluorescence staining of PKC isoforms in HT-3 cells

These representative pictures show the intracellular localization of PKC-α (*A*), -β (*B*) and -γ (*C*), respectively, in isotonic conditions at various time periods after 210 mosmol l^{-1} hypotonic solution perfusion. The observations showing the effects of hyposmolarity on β- and γ-PKC are shown 15 and 30 min after hypotonic solution perfusion. Scale bar, 10 μm. For details, see Methods.

Fig. 1, injection of anti-α (25 ng ml^{-1}) slowed down the process of RVD: cell volume at 20 min was 1·62 ± 0·07 ($n = 15$) of initial cell volume compared with a 20 min volume of 1·39 ± 0·03 for the control measurement in the absence of antibody. After 40–50 min of hypotonic perfusion, the HT-3 cells injected with anti-α had not returned to their initial cell size, but reached a plateau which was 30% higher compared with the HT-3 cells without antibody treatment. On increasing the anti-α concentration further to 250 or 2500 ng ml^{-1}, the disturbance of RVD became more evident; however, the inhibitory effect between these two concentrations was not significantly different ($P > 0·05$, unpaired t test). The RVD process was not significantly affected by either anti-β or anti-γ injection (Fig. 1; $P > 0·05$, unpaired t test).

Immunohistochemistry

To confirm the involvement of PKC-α in RVD responses further, the subcellular localization of the different PKC isoforms and the translocation in response to hyposmolarity have been studied with immunocytochemistry in immunofluorescence microscopy. As shown in Fig. 2*A*, in HT-3 cells under isotonic conditions PKC-α shows a diffuse, homogeneous cytosolic staining pattern. After a 5 min hypotonic perfusion, PKC-α seemed to aggregate in certain portions of the cytosol (not illustrated). The aggregation of PKC-α intensified (10 min, middle panel, Fig. 2*A*) and an increase in membrane PKC-α (10 min, right panel, Fig. 2*A*) was evident in approximately two thirds of cells 10 min after hypotonic exposure. Membrane PKC-α tended to increase with time and reached peak intensity in 30 min, with an almost complete disappearance of the PKC-α from the cytosol. The distribution of PKC-α was mainly cytosolic when observed after a 60 min hypotonic exposure.

PKC-β and PKC-γ appeared localized in the membrane and cytosol fractions both in control and in HT-3 cells 15 or 30 min after hypotonic perfusion. Cell swelling did not change the subcellular distribution of these PKC isoforms appreciably (Fig. 2*B*, PKC-β; Fig. 2*C*, PKC-γ).

Western blot analysis

The intracellular localization of α, β and γ PKC isoforms has also been investigated with Western blots (Figs 3, 4 and 5).

The behaviour of PKC-α is shown in Fig. 3. PKC-α appears as double bands, and most of this isoform is localized in the cytosolic fraction. However, the densitometric analysis of the change indicated that cell swelling induced a markedly lowered PKC-α signal, especially the phosphorylated form of PKC-α (upper bands), in the cytosolic fraction (Fig. 3*A*). Concomitantly, after 10 min hypotonic treatment a marked increase in PKC-α associated with the particulate fraction

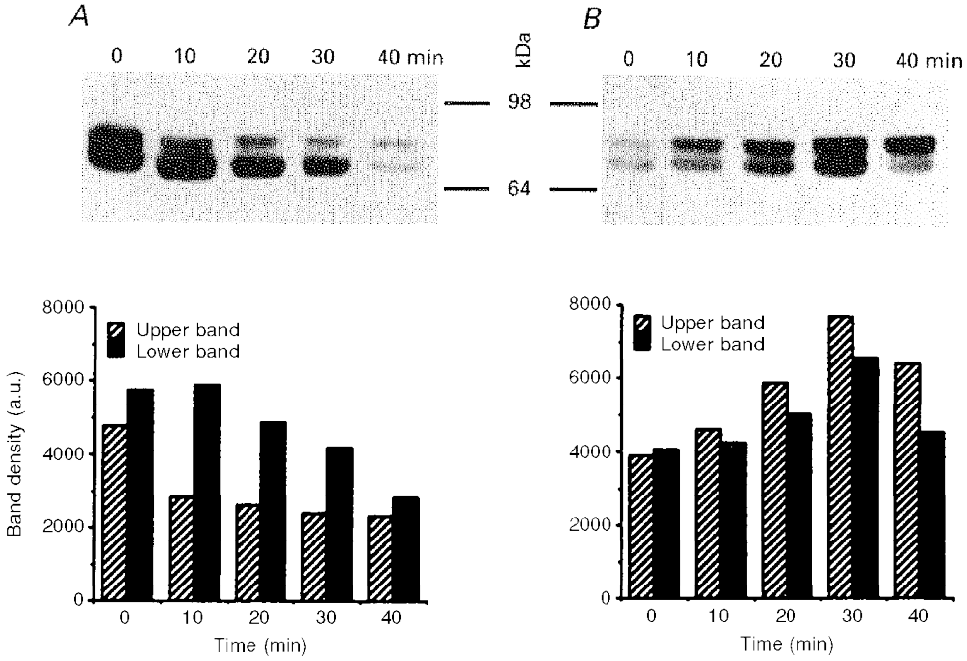

Figure 3. Western blot and quantification of PKC-α in HT-3 cells

HT-3 cells were exposed to isotonic solution (290 mosmol l^{-1}) and then lysed immediately or after hypotonic solution (210 mosmol l^{-1}) perfusion for 10–40 min. After separation of cytosolic (*A*) and particulate (*B*) fractions, 15 μg of each protein from the two fractions was denatured in SDS lysis buffer and loaded into 10% SDS–polyacrylamide gels. Blotting and immunodetection with PKC-α antibody were then carried out. Histograms represent the densitometric reading of the corresponding bands. For details of the assay see Methods. A typical experiment was shown here, which had been repeated five times with comparable results. The data in lane 1, panel *B* has been cut and then pasted from a different lane of the same film. a.u., arbitrary units.

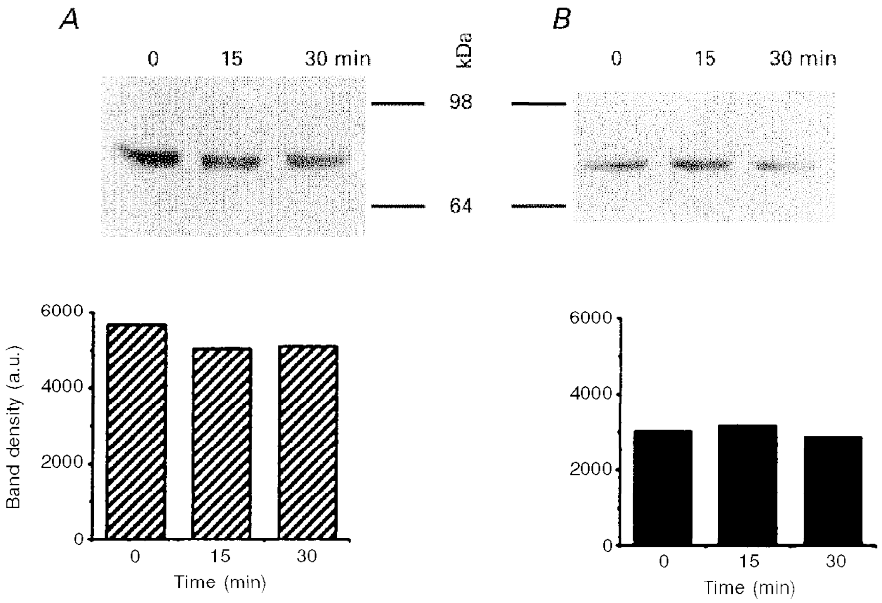

Figure 4. Western blot and quantification of PKC-β in HT-3 cells

HT-3 cells were exposed to isotonic solution (290 mosmol l^{-1}) and then lysed immediately or after hypotonic solution (210 mosmol l^{-1}) perfusion for 15 or 30 min. After separation of cytosolic (*A*) and particulate (*B*) fractions, 15 μg of each protein from the two fractions was denatured in SDS lysis buffer and loaded into 10% SDS–polyacrylamide gels. Blotting and immunodetection with PKC-β antibody were then carried out. Histograms represent the densitometric reading of the corresponding bands. For details of the assay see Methods. A typical experiment is shown here, which had been repeated five times with comparable results.

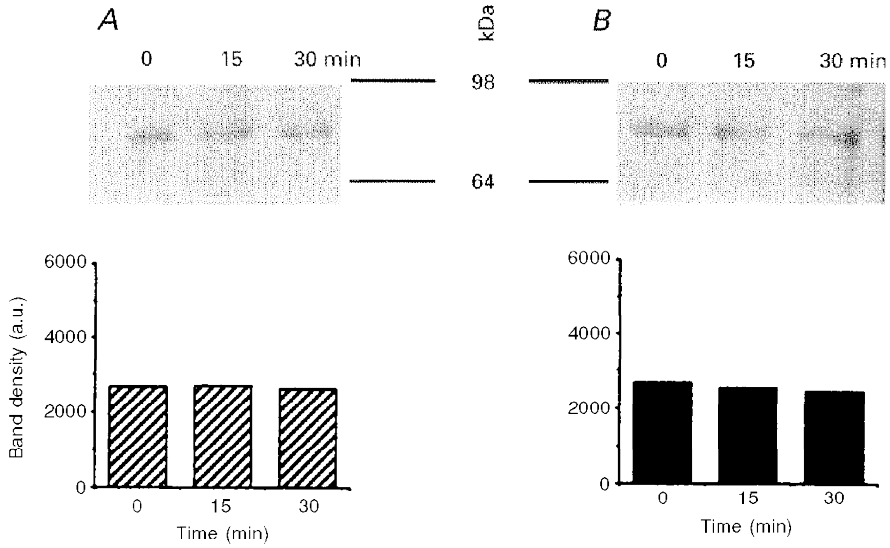

Figure 5. Western blot and quantification of PKC-γ in HT-3 cells

HT-3 cells were exposed to isotonic solution (290 mosmol l^{-1}) and then lysed immediately or after hypotonic solution (210 mosmol l^{-1}) perfusion for 15 or 30 min. After separation of cytosolic (*A*) and particulate (*B*) fractions, 15 μg of each protein from the two fractions was denatured in SDS lysis buffer and loaded into 10% SDS–polyacrylamide gels. Blotting and immunodetection with PKC-γ antibody were then carried out. Histograms represent the densitometric reading of the corresponding bands. For details of the assay see Methods. A typical experiment is shown here, which had been repeated five times with comparable results.

was found. The membrane positivity increased with time in both phosphorylated and dephosphorylated PKC-α. The membrane signal peaked after a 30 min period, and then showed a decrease in the level of PKC-α, especially the dephosphorylated PKC-α, after 40 min exposure to hyposmolarity (Fig. 3*B*).

Under isotonic conditions, PKC-β appeared more intense in the cytosolic than in the particulate fraction, while PKC-γ levels were less intense than PKC-α and PKC-β but seemed to localize equally in both fractions. Neither PKC-β nor PKC-γ showed marked changes in distribution or intensity 15 and 30 min after hypotonic exposure (Fig. 4, PKC-β; Fig. 5, PKC-γ).

Characterization of volume-sensitive chloride currents

Figure 6*A* shows a representative patch-clamp recording obtained from HT-3 cells. When the cells were whole-cell clamped in the isotonic solution, the basal chloride currents in response to a voltage range of −80 to +40 mV were steady and small. However, a current with mild outward rectification was induced 3–5 min after perfusing the cells with hypotonic solution in 92/100 cells. The current reached maximal amplitude within the subsequent 5 min, and was sustained at a plateau, responding to hypotonic stress. Hypotonicity elicited an increase in current from 0·29 ± 0·19 and −0·2 ± 0·1 nA to 5·25 ± 0·25 and −4·75 ± 0·63 nA at +40 and −80 mV, respectively. It is noteworthy that both the absolute amplitude and the degree of outward rectification of the volume-sensitive currents varied (compare, for example, Fig. 6*A* with Fig. 7*B*), suggesting the involvement of more than one type of chloride channel. The hypotonicity-induced current reversed at −4·8 ± 2·1 mV ($n = 92$), which was close to the theoretical reversal potential for chloride ions. A Cl^- channel blocker, NPPB, inhibited the volume-sensitive chloride current in a dose-dependent manner. The effect of NPPB was reversible, of rapid onset, and both the outward and inward currents were equally inhibited (Fig. 6*A*). Figure 6*B* demonstrated the dose–response curves of various chloride channel blockers on the inhibition of volume-sensitive currents measured at +40 mV. The concentration that attained the half-maximal inhibition was 50, 100 and 500 μM for NPPB, SITS and DIDS, respectively. To clarify further whether this current was predominantly carried by chloride ions, the external chloride concentration in hypotonic solution was replaced with an equimolar concentration of sodium gluconate. Figure 6*C* shows the relationship between the reversal potential and the extracellular concentration of chloride ions in HT-3 cells. When the chloride concentration inside the pipette solution was kept at 135 mM and the extracellular chloride concentration was reduced by replacement with sodium gluconate, the reversal potential for the hypotonicity-induced current shifted to more positive values.

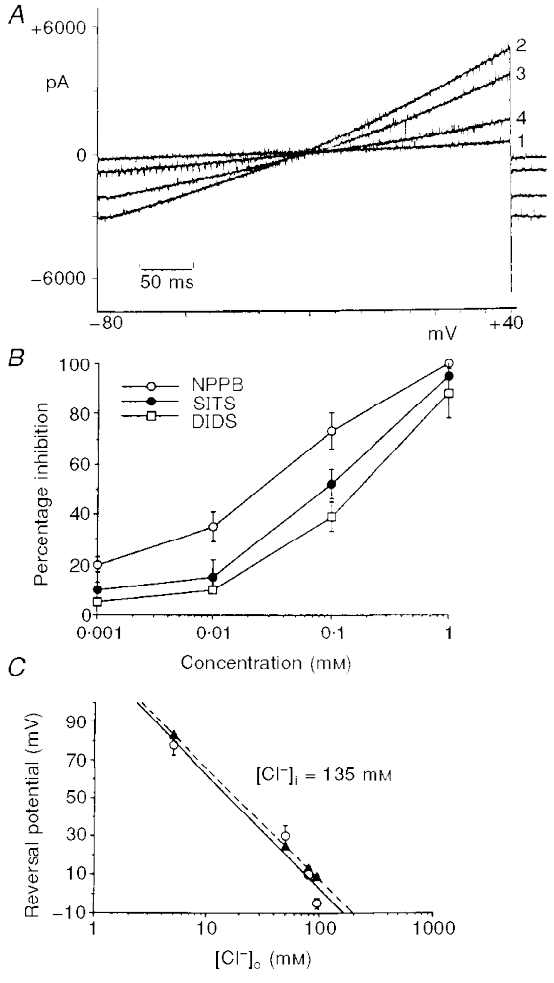

Figure 6. Characterization of volume-sensitive chloride currents in HT-3 cells

A, a Cl^- channel blocker, NPPB, with a dose-dependent manner inhibited the volume-sensitive Cl^- current in HT-3 cells. The membrane potential was held at −80 mV and the ramp command pulse from −80 to +40 mV was employed. 1, basal membrane current recorded under isotonic conditions (290 mosmol l^{-1}); 2, current recorded after perfusion with hypotonic solution (210 mosmol l^{-1}); 3 and 4, currents recorded after adding 10 or 100 μM NPPB to the perfusing hypotonic solution. *B*, dose–response relationships of Cl^- channel blockers for the percentage of inhibition of volume-sensitive Cl^- current measured at +40 mV. Each point is mean ± S.E.M. ($n = 6$). *C*, the reversal potential for the volume-sensitive Cl^- current at various extracellular Cl^- concentrations ($[Cl^-]_o$). The line was well fitted by the linear regression analysis. The dashed line represents the theoretical values of reversal potential in different Cl^- concentrations estimated by the Goldman–Hodgkin–Katz equation.

For instance, when the extracellular chloride concentration was reduced to 80 and 5 mM, the reversal potential was significantly changed to 8 ± 5 and 75 ± 6 mV, respectively ($n = 6$). These findings indicated the strong dependence of changes of ionic currents on extracellular chloride concentration. In other words, the more the external chloride concentration is reduced, the more the driving force on the chloride ion through the open chloride channels is increased. A linear correlation was obtained between the values of the extracellular chloride concentration and those for reversal potential (Fig. 6*C*). Altogether, these results suggest that hypotonicity activates volume-sensitive Cl^- currents in HT-3 cells.

Signal transduction pathways

To assess possible signal transduction pathways involved in the activation of volume-sensitive chloride channels, the following reagents were used: neomycin, a PLC blocker; 4-bromophenacyl bromide (pBPB), a phospholipase A_2 inhibitor; erbstatin analogue, a tyrosine kinase blocker; staurosporine and H7, PKC blockers; PMA, a PKC activator; protein kinase A inhibitor (PKI), a PKA inhibitor; IBMX, a cAMP phosphodiesterase inhibitor, causing an increase in endogenous cAMP and thus indirectly causing activation of PKA; and di-butyryl cAMP (db-cAMP), a cell membrane-permeant c-AMP analogue. When 1 mM neomycin was added to the pipette solution, it had no effect on the basal currents in the isotonic condition. However, neomycin blocked the hypotonicity-induced chloride current in 15/15 cases (Fig. 7*A*). Similarly, as depicted in Fig. 7*B*, the hypotonicity-induced Cl^- currents (trace 2) were reversibly inhibited by changing the perfusing medium to the isotonic condition (trace 3), or by adding PKC blockers such as 1 μM staurosporine (trace 4) or 100 μM H7 (data not shown) into the perfusing hypotonic solution. Staurosporine and H7 almost completely blocked volume-sensitive chloride

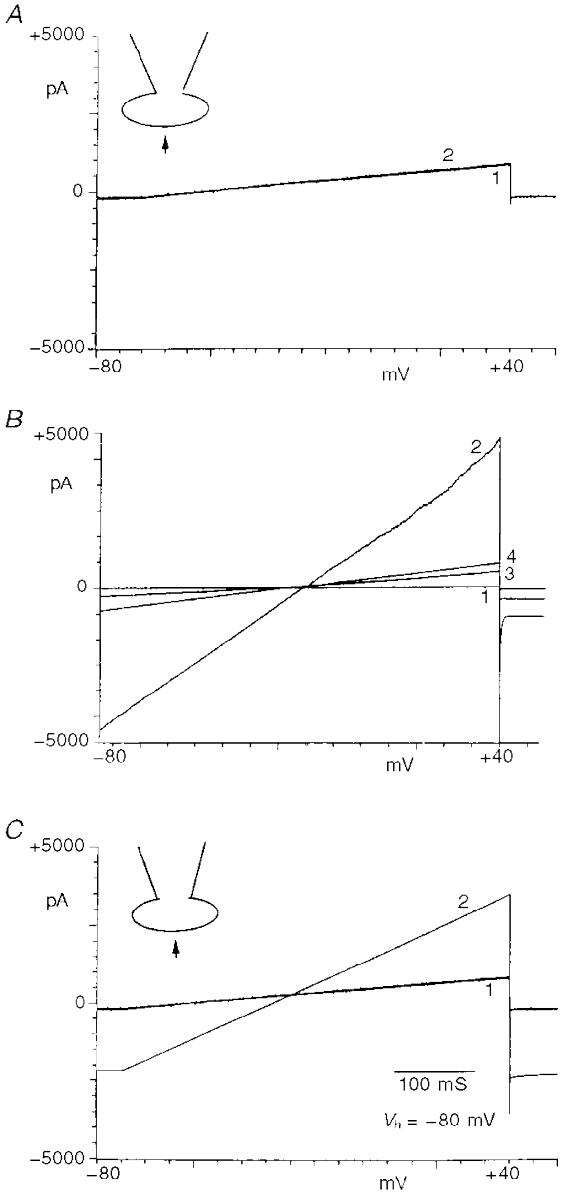

Figure 7. Effects of neomycin, staurosporine and the combined use of neomycin and PMA on volume-sensitive chloride currents in HT-3 cells

A, effect of neomycin, a blocker of phospholipase C, on volume-sensitive Cl^- current in HT-3 cells. 1, basal membrane current recorded in isotonic conditions; 2, current recorded after perfusion with hypotonic solution when 1 mM neomycin was added to the pipette solution. *B*, effect of staurosporine, a blocker of protein kinase C, on volume-sensitive Cl^- current in HT-3 cells. 1, basal membrane current recorded in isotonic conditions; 2, current recorded after perfusion with hypotonic solution; 3, current recorded after perfusing solution was shifted from hypotonicity to isotonicity; 4, current recorded after adding 1 μM staurosporine to the perfusing hypotonic solution. *C*, interaction of neomycin and PMA on volume-sensitive Cl^- current in HT-3 cells. 1, current recorded after perfusing with hypotonic solution when 1 mM neomycin was added to the pipette solution; 2, current recorded after adding 0·1 μM PMA to the hypotonic solution and 1 mM neomycin to the pipette solution; V_h, holding potential.

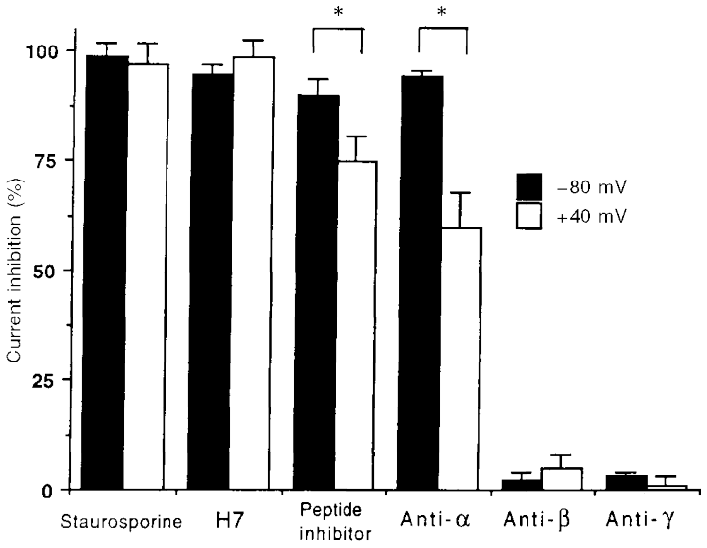

Figure 8. Effects of PKC blockers and isoform-specific PKC antibodies on volume-sensitive chloride currents in HT-3 cells

The effects of PKC blockers such as staurosporine and H7, PKC peptide inhibitors, and isoform-specific PKC antibodies on volume-sensitive chloride currents measured at −80 and +40 mV. The *y*-axis is the percentage inhibition of volume-sensitive chloride currents induced by each blocker. Each bar represents the mean ± S.E.M. ($n = 16$); * significance level of $P < 0{\cdot}05$ (paired *t* test). Peptide inhibitor is the PKC inhibitor as mentioned in Methods.

currents at −80 and +40 mV, and the outward and inward currents were equally sensitive to these blockers (for staurosporine, 98 ± 5% inhibition at −80 mV *vs.* 95 ± 7% inhibition at +40 mV, $P > 0{\cdot}05$, paired *t* test; for H7, 93 ± 5% inhibition at −80 mV *vs.* 99 ± 7% inhibition at +40 mV, $P > 0{\cdot}05$, paired *t* test, $n = 16$ cells).

Further experiments were performed to investigate the interaction between PLC and PKC in the modulation of volume-sensitive chloride currents. The results showed that the PKC activator PMA could reverse the inhibitory effect of neomycin on Cl^- currents. As shown in Fig. 7*C*, 1 mM neomycin in the pipette solution suppressed the activation of chloride currents (trace 1). However, the volume-sensitive currents could be activated within 5 min when 0·1 μM PMA was added into the hypotonic medium combined with 1 mM neomycin in the pipette solution (trace 2). This prompted speculation that conventional PKCs seemed to play a role in the activation of this current. Therefore, we tested the effects of PKC peptide inhibitor and three conventional PKC antibodies, i.e. α, β and γ, on the activation of volume-sensitive chloride currents. The differential effects of various PKC inhibitors are compared and summarized in Fig. 8. By the inclusion of either 1 μM PKC peptide inhibitor or 250 ng ml^{-1} anti-PKC-α in the pipette solution, the chloride currents typically observed in the hypotonic stress were significantly suppressed. It is worth noting that the inward current was more sensitive to peptide inhibitor or anti-PKC-α than was the outward current, a pattern quite different from those induced by staurosporine and H7. The inhibition of volume-sensitive chloride currents by peptide inhibitor was 90 ± 4 and 75 ± 6% at −80 and +40 mV, respectively, a significant difference (paired *t* test, $n = 16$ cells). There was no change in these observations over a 15 min period. Similarly, the percentage of inhibition induced by anti-PKC-α was 95 ± 1 and 63 ± 8% at −80 and +40 mV, respectively

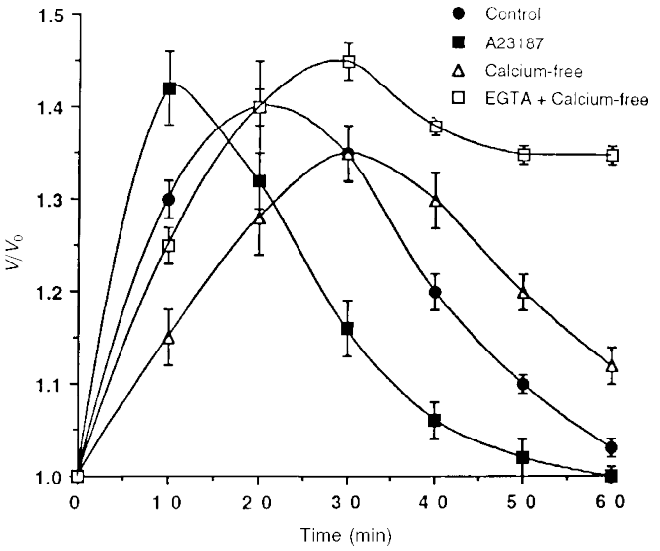

Figure 9. Role of calcium on cell volume regulation of HT-3 cells on exposure to 210 mosmol l^{-1} hypotonic stress

The *y*-axis (V/V_0) depicts the cell volume at the indicated times divided by the cell volume at zero time. Each point represents mean ± S.E.M. ($n = 20$ cells) from three separate experiments. A23187 (10 μM) was added to the hypotonic medium. Calcium-free medium was the hypotonic medium without addition of calcium. EGTA was transferred into cells by microinjection. EGTA at 1 mM had minimal effect on RVD process (data not shown).

($P < 0{\cdot}05$, paired t test, $n = 16$ cells). In contrast, anti-PKC-β and anti-PKC-γ had no significant effect on the activation of volume-sensitive chloride currents in HT-3 cells ($n = 16$, Fig. 8).

When HT-3 cells were exposed to the hypotonic solution, the activation of volume-sensitive Cl^- currents was not significantly affected by the addition of pBPB, erbstatin analogue, PKI, IBMX or db-cAMP. The data suggest that none of the protein kinase A, cAMP, phospholipase A_2 or tyrosine kinase pathways are implicated in the regulation of volume-sensitive Cl^- channels in HT-3 cells.

Role of calcium

Calcium plays a controversial role in cell volume regulation in different types of epithelial cells (Hoffmann & Dunham, 1995; Okada, 1997). A series of experiments were therefore carried out to determine if Ca^{2+} was required for the RVD responses or the activation of the volume-sensitive chloride currents in HT-3 cells.

When a calcium ionophore A23187 at 10 μM was added to the hypotonic medium, the time course of RVD was shortened significantly. HT-3 cells reached the maximal cell volume at about 11 ± 3 min and the cell volume returned to the steady state at about 40 min. In contrast, without A23187 treatment, the cell reached maximal size at 20 ± 5 min and returned to the original cell size at about 50 min after exposure to hypotonic stress (Fig. 9). Microinjection of either 0·1 or 1 mM EGTA did not significantly affect the RVD responses (not shown). However, the time required to reach maximal cell volume was significantly prolonged (30 ± 4 min) when HT-3 cells were exposed to nominally Ca^{2+}-free hypotonic medium. RVD was inhibited to a further extent by microinjection of 10 mM EGTA and

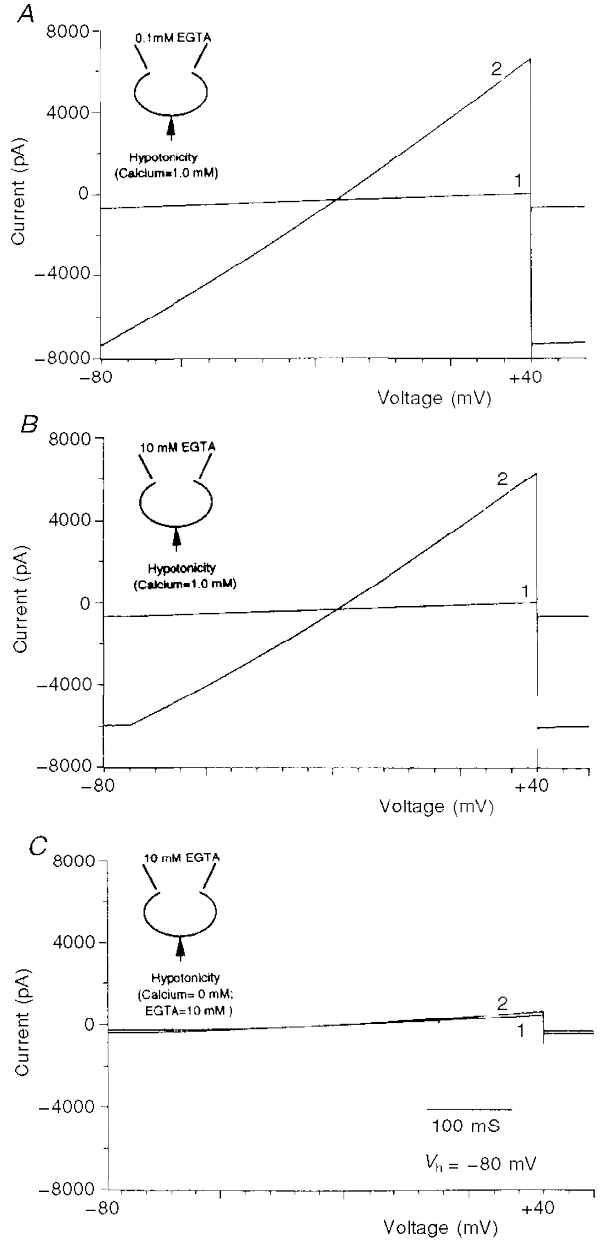

Figure 10. Effect of calcium depletion on the activation of volume-sensitive Cl^- current in HT-3 cells

A, 1, basal membrane current recorded in isotonic conditions. 2, current recorded in the hypotonic perfusing solution with 0·1 mM EGTA in the pipette solution. *B*, 1, basal membrane current recorded in isotonic conditions; 2, current recorded in the hypotonic perfusing solution with 10 mM EGTA in the pipette solution. *C*, 1, basal membrane current recorded in isotonic conditions; 2, current recorded in the calcium-free hypotonic solution with 10 mM EGTA in the pipette solution.

subsequent superfusion with Ca^{2+}-free hypotonic medium (Fig. 9).

In the patch-clamp studies, when the pipette solution contained 0·1 mM EGTA and no calcium, the volume-sensitive chloride currents were persistently activated in 15/15 cells (Fig. 10*A*). To buffer the internal Ca^{2+} further, the concentration of EGTA in the pipette solution was raised to 10 mM. Cells were incubated in the isotonic solution ($[Ca^{2+}] = 1·5$ mM) for 20 min and then were exposed to the hypotonic solution ($[Ca^{2+}] = 1·0$ mM). Under these conditions, hypotonicity still activated the volume-sensitive chloride currents ($n = 7$) and there was no significant difference in the chloride conductance compared with the previous condition (Fig. 10*B*). However, the combined use of 10 mM EGTA and calcium-free solutions significantly inhibited the chloride conductance. Cells were incubated in calcium-free isotonic solution for 20–30 min, and were subsequently exposed to calcium-free hypotonic solution, while 10 mM EGTA was added in the pipette solution. Under these conditions, no volume-sensitive current was elicited ($n = 8$, not illustrated). To be certain that extracellular calcium was removed, experiments were repeated in calcium-free isotonic and then hypotonic solutions to which 10 mM EGTA had been added, while 10 mM EGTA was added to the pipette solution. Under these circumstance, no current was activated by hypotonicity ($n = 8$, Fig. 10*C*). Taken together, these experiments indicated that RVD responses and the activation of volume-sensitive chloride currents depended on extracellular calcium or the process of calcium influx.

DISCUSSION

Our data suggest the involvement of PKC-α and extracellular calcium in the RVD responses and the signal transduction pathways underlying the activation of volume-sensitive chloride channels in human cervical cancer HT-3 cells. Microinjection of anti-PKC-α significantly prolonged the time course of RVD responses, and translocation and phosphorylation of PKC-α from cytosol to membrane during cell swelling were demonstrated by immunocytochemical staining and Western blots. Furthermore, the increased intensity of membrane PKC-α was in concert with cell swelling, while its decrease after prolonged hypotonic perfusion corresponded to the recovery of cell volume. This clearly indicates hyposmolarity-induced activation of PKC-α in HT-3 cells. The present study also shows that the PKC involved in the signalling of volume-sensitive chloride channels in HT-3 cells is a downstream component of phosphatidylinositol-specific PLC signalling pathways. Further experiments show that PKC-α is responsible for the signalling events involved in the volume-sensitive currents.

An increase in cell volume activates a Cl^- current in most mammalian cells (Nilius *et al.* 1994). This current is involved in a variety of cellular functions, such as the maintenance of a constant cell volume, pH regulation, and control of membrane potential (Hoffmann & Dunham, 1995; Haussinger, 1996). It might also play a role in the regulation of cell proliferation and in the processes that control transition from proliferation to differentiation (Nilius *et al.* 1996; Phipps *et al.* 1996). In a series of studies, we have shown that the expression of volume-sensitive Cl^- channels is associated with human cervical carcinogenesis (Chou *et al.* 1995; Shen *et al.* 1996). The volume-sensitive Cl^- channels in cervical cancer cells could mediate the transport of taurine, and pertussis toxin-sensitive G protein is linked with the activation of this transport mechanism (Chou *et al.* 1997). The present study has shown that volume-sensitive chloride channels are regulated by the PLC signalling with downstream PKC activation which is comparable to the case in RVD responses (Shen *et al.* 1998). Further studies show the implication of PKC-α in the modulation of both RVD responses and volume-sensitive currents. Moreover, in the pharmacological studies, the potency of chloride channel blockers was NPPB > SITS > DIDS. The order of potency was consistent with our previous results showing the inhibitory effects of these blockers on volume-activated taurine transport (Chou *et al.* 1997), volume-sensitive Cl^- currents in the primary culture cells of cervical cancer (Shen *et al.* 1996) or in the cervical cancer cell line CX (Chou *et al.* 1995), and RVD responses in HT-3 cells (authors' unpublished observations). Altogether, these findings strongly suggest that there is a common cellular signalling pathway in the regulation of RVD responses and volume-sensitive Cl^- channels. Thus, we propose that this Cl^- conductance plays the pivotal role leading to RVD in human cervical cancer cells.

PKC is involved in basic cellular functions, such as regulation of growth, differentiation and gene expression (Nishizuka, 1995). PKC-α is the most widely expressed PKC in most types of cells. The overexpression of PKC-α can lead to an enhanced growth rate, and potent inhibitors of PKC are shown to have cytostatic and chemosensitizing properties (Powis, 1992). The overexpression of different PKC isoforms may induce opposite effects on growth, anchorage dependence, and tumorigenicity (Mischak *et al.* 1993). Furthermore, differential localization of PKCs or different amounts of PKC-binding proteins and substrates have been observed in normal and transformed cells, implicating the role of PKC in carcinogenesis (Hyatt *et al.* 1994; Chapline *et al.* 1996). Our previous study also showed that the cell volume regulation in cervical cells with different tumour potential is possibly mediated by different PKC isoforms. It will be interesting to study the signalling pathways in non-cancerous cells such as HPV-immortalized cell lines and normal cervical epithelia, and to compare the differences in signalling pathways underlying RVD responses in these cervical cells. Once the signalling events in these cervical cells can be understood better, the correlation between PKC and human cervical carcinogenesis could be examined further.

Previous reports show that PKC has varying effects on RVD responses or volume-sensitive chloride channels in

different cell types. The PKC regulation of volume-sensitive Cl^- channels or RVD has been observed in hepatocytes of guinea-pig (Koumi *et al.* 1995), NIH3T3 cells expressing P-glycoprotein (Hardy *et al.* 1995), proximal tubule cells of *Rana temporaria* (Robson & Hunter, 1994*a*,*b*), ciliary epithelium (Coca-Prados *et al.* 1995), pancreatic duct cells (Verdon *et al.* 1995), and skate erythrocytes (Musch & Goldstein, 1990). In contrast the ClC-2 or ClC-3 currents were inhibited by activation of PKC (Fritsch & Edelman, 1994; Kawasaki *et al.* 1994). Moreover, PKC is not essential for the regulation of volume-sensitive Cl^- channels or RVD in human (Nilius *et al.* 1994) or bovine endothelial cells (Szücs *et al.* 1996), HeLa (Hardy *et al.* 1995), rat oesteoblast-like, and rat hepatoma cells (Gosling *et al.* 1995; Schliess *et al.* 1995). However, these studies emphasized the pharmacological modulation of RVD responses or chloride currents, and little information is available concerning identification of the specific type of PKC involved. Our present study, by using isoform-specific PKC antibodies, is able to provide the direct indication of cell-swelling-induced translocation and phosphorylation of PKC-α in cell membrane with concomitantly decreased expression of PKC-α in the cytosol. Phosphorylation in kinases is often linked to the processing (Newton, 1997) or modulation of kinase activity (Galabru & Hovanessian, 1987; Hunter, 1987), and the proteolytic activation and degradation of PKC (Ohno *et al.* 1990; Pears & Parker, 1991). It is also known to increase its affinity for calcium, phorbol esters and protein substrates (Huang *et al.* 1986; Mochly-Rosen & Koshland, 1987). Moreover, several reports suggest a post- or co-translational phosphorylation of PKC-α prior to its activation (Borner *et al.* 1989; Pears *et al.* 1992). Activation of PKCs will result in phosphorylation of the substrates. However, this study does not provide evidence that PKC phosphorylates the chloride channel. Alternatively, PKC may phosphorylate certain binding proteins that link PKC to the channel. Further studies are required to determine the effect of phosphorylation on the activity state of PKC in HT-3 cells.

We have shown here that in HT-3 cells both RVD and swelling-induced activation of chloride current are insensitive to changes in intracellular Ca^{2+} but depend on external calcium or calcium influx. In this study, promoting Ca^{2+} influx by adding A23187 accelerated while calcium-free medium blocked cell volume recovery, suggesting the dependency of RVD on extracellular Ca^{2+}. In contrast, removal of intracellular Ca^{2+} by microinjection of EGTA does not affect the RVD responses. Similar results were obtained from the patch-clamp studies. Even though EGTA may not totally remove intracellular Ca^{2+}, however, the sensitivities of RVD and Cl^- channels to the changes of extracellular or intracellular Ca^{2+} are distinctly different. Altogether, the data suggest a role for Ca^{2+} entry, and a permissive role only for intracellular Ca^{2+}. Similar results have been presented from proximal renal tubule cells (McCarty & O'Neil, 1992), pancreatic duct cells (Verdon *et al.* 1995), and distal bright convoluted tubule (DCTb) cells (Rubera *et al.* 1997), and their data also suggest the role of Ca^{2+} channels for a swelling-activated Ca^{2+} entry. Our previous data show that the Ca^{2+} channel blocker verapamil interferes with the volume-sensitive chloride channels in cervical cancer cells (Chou *et al.* 1995), suggesting the presence of similar Ca^{2+} channels in HT-3 cells.

Our previous and present studies provide evidence that can begin to outline the pathways linking cell swelling to the activation of Cl^- current in human cervical cancer cells. The following mechanism is proposed. Cell membranes sense the signal of hypotonic stress and activate the G protein-coupled receptors. These receptor-induced signals presumably initiate the hydrolysis of inositides by PLC and subsequently the activation of PKC. Meanwhile, cell swelling activates a Ca^{2+} influx pathway. This may allow Ca^{2+} to move into cells down the electrochemical gradient, and stimulates the Ca^{2+}-dependent PKC. Activation of PKC accelerates RVD and activates the volume-sensitive Cl^- channels. The net effect of the above reactions is the loss of intracellular Cl^-, presumably in conjunction with a cation. Finally, the recovery of cell volume happens. Further experiments are needed to determine the interaction between the Ca^{2+} influx pathway and the G protein–PLC–PKC signalling pathways in the cell volume regulation of HT-3 cells.

In conclusion, we demonstrate here that PKC-α regulated both RVD responses and volume-sensitive Cl^- channels in HT-3 cells. Further studies are under way to elucidate the role of PKCs in cervical carcinogenesis.

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Acknowledgements

This work was supported by grant NSC-88-2314-B006-088 (National Science Council, Taiwan), and grants from the National Cheng Kung University Medical College and the Hospital, Tainan, Taiwan.

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