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Selective modulation of P-glycoprotein's ATPase and anion efflux regulation activities with PKC α and PKC ε in Sf9 cells

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Abstract The modulation of P-glycoprotein's (Pgp) ATPase activity and its ability to regulate swelling-activated ^{125}I efflux, by PKC α and PKC ε, was examined in insect cells. Recombinant baculovirus was used to express human Pgp in Sf9 cells and Pgp was also co-expressed with either PKC α or PKC ε. ATPase assays showed the enzyme activity of Pgp to be elevated during co-expression with the Ca^{2+} dependent isoform PKC α, but not with the Ca^{2+} independent variant PKC ε. Furthermore, neither isoform, when co-expressed with Pgp, altered the swelling-activated efflux of ^{125}I from Sf9 cells. However, in cells co-expressing Pgp/PKC (α or ε), pre-treatment with the phorbol ester TPA significantly reduced the swelling-activated ^{125}I efflux with both PKC isoforms. Our results suggest that phosphorylation with the Ca^{2+} independent variant PKC ε does not regulate the ATPase activity of Pgp and that stimulation of PKC with TPA alters the swelling-activated efflux of anions from insect cells expressing Pgp.

Key words P-glycoprotein · PKC · Chloride channels · Baculovirus · Cancer · MDR

Abbreviations *ATP* Adenosine triphosphate · *CHO* Chinese hamster ovary · *Pgp*: P-glycoprotein · *PKC* Protein kinase C · *MDR* Multidrug resistance · *RVD* Regulatory volume decrease · *Sf Spodoptera frugiperda* · *TPA* 12-O-tetradecanoylphorbol-13-acetate

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Introduction

P-Glycoprotein (Pgp) is the protein product of the multidrug resistance (mdr) gene and is implicated in resistance to a wide range of hydrophobic chemotherapeutic drugs in cancer cells. This membrane protein has an apparent molecular weight of 140 kDa, which increases to 170 kDa upon glycosylation in cells. The protein has been cloned in bacterial, yeast, insect and mammalian expression vectors (reviewed in [1, 2]). The protein is a member of the ATP binding cassette (ABC) transporter superfamily and shows ATPase activity associated with its transport function (reviewed in [3, 4]). Over-expression of Pgp in mammalian cells has been shown to be associated with volume-sensitive anion-conductance ($I_{Cl,swell}$). Although it was originally proposed that Pgp functioned as a volume-sensitive anion channel [5], this proposal was soon contradicted [6, 7]. It is now believed that Pgp serves as a regulator of volume-sensitive anion channel(s), rather than having inherent channel activity reviewed in [8, 9]. Furthermore, phosphorylation of Pgp may function as a molecular switch separating the transport and channel regulation activities [10, 11]. However, it has been proposed that phosphorylation of Pgp with PKC does not effect drug transport [12] or volume-sensitive Cl^- channel activities [13, 14].

Protein phosphorylation is one of the most characterised post-translational modifications and is known to regulate enzyme activities as well as mediating signal transaction in eukaryotes [15]. Many transcription factors, including the ligand-dependent steroid/thyroid receptor family, nucleic acid interactive and membrane proteins, are phosphorylated and are regulated through the action of protein kinases [16, 17, 18]. Cell cycling depends on the serial phosphorylation and dephosphorylation of the cyclins and the kinases they regulate [19]. P-Glycoprotein possesses 35 potential sites for PKC/PKA phosphorylation, uniformly distributed throughout its primary sequence. However, only four serines (661, 667, 671 and 683) are known to be phosphorylated

by PKA and PKC in vitro, of which only three serines (661, 667 and 671) are actually phosphorylated in vivo [20, 21]. Individual phosphorylation of these serines may serve distinct functions and collective phosphorylation of all three may serve another unique role.

PKC is a family of closely related protein kinase isoforms characterised by their dependence on phospholipid and diacylglycerol [22]. To date, eleven PKC isoforms have been identified and classified into three groups based on their structure and co-factor requirement [23, 24]. These groups are: the calcium dependent group (PKC α, βI, βII and γ), the calcium independent group [PKC δ, ε, η, θ, μ and (L)] and a third group that does not reportedly respond to phorbol esters [PKC ξ and λ(I)]. Information concerning the expression and specific role of individual PKC isoforms in the MDR phenotype is limited. Previous studies have shown the MDR phenotype to be associated with a 10-fold increase in calcium dependent (due to increased PKC α expression) and 10-fold decrease in calcium independent (due to a decrease in PKC δ and ε expression) PKC activity in human breast cancer (MC-7) cells [25]. Furthermore, phosphorylation of Pgp with PKC α was shown to stimulate its ATPase activity when co-expressed in Sf9 cells [26]. In the present study baculovirus expression was used to compare the effect of co-expressing Pgp with either PKC α or PKC ε on Pgp's ATPase activity in Sf9 cell membranes. Additionally, we examined the effect(s) of Pgp/PKC co-expression on the volume-sensitive ^{125}Iodide efflux from Sf9 cells. Our results indicate that, unlike PKC α, PKC ε failed to stimulate Pgp's ATPase activity. Furthermore co-expression of Pgp with PKC reduced the volume-sensitive ^{125}I efflux from Sf9 cells that had been pre-treated with TPA.

Materials and methods

The Baculovirus Expression Kit (BacPAK) was purchased from Clontech. Recombinant baculovirus expressing PKC and polyclonal antibodies recognising PKC α or PKC ε were a generous gift from Dr. P. Parker (ICRF, London). Cell culture media were from GIBCO-BRL, and the ECL detection kit was from Amersham. ^{125}Iodide (carrier free) was also from Amersham. The BCA protein determination kit was from Bio-Rad. The anti-Pgp monoclonal antibody was from Centircor (PA, USA). All other chemicals were purchased from Sigma.

Construction of MDR-1 recombinant baculovirus

Sf9 cells were co-transfected with Bsu36I digested AcMNPV genomic DNA and the pBacPAK9 transfer vector (Clontech) with the human MDR1 cDNA [5] inserted in the BamHI site. The cDNA encoded six histidine residues at the N-terminus, preceded by a thrombin site. Recombinant viruses were isolated by three rounds of plaque purification and grown to high titre (5×10^8 pfu/ml) following standard procedures [27].

Sf9 cell culture and PGP/PKC expression

Sf9 cells were cultured at 27 °C in TC-100 medium (GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS) as described elsewhere [28]. Monolayer (10–12 ml of medium containing 5×10^7 cells) or suspension cultures (50–100 ml of medium with 1×10^6 cells/ml) of Sf9 cells in log-phase were infected with the recombinant virus AcMNPV/hisMDR1 at a multiplicity of infection of 10 or with a 1:1 mixture of AcMNPV/hisMDR1 and AcMNPV/PKC (α or ε) at a multiplicity of infection of 5 for each. Cells were harvested for membrane preparation 48–60 h post infection. This represented optimal expression time for Pgp, without causing significant cell lysis.

Preparation of Sf9 membranes

Insect cell membranes from cells infected with the wild-type baculovirus or baculovirus expressing Pgp and/or PKC were prepared using a cell disruption chamber pressurised to 1000–1500 psi under nitrogen. Briefly, harvested cells were pelleted and resuspended in 20 ml buffer 1 (10 mM Tris-HCl pH 7.4, 0.25 M sucrose and 0.2 mM $CaCl_2$). The cells were then disrupted in the nitrogen chamber twice for 15 min each time. The cell suspension was diluted to 50 ml in buffer 2 (10 mM Tris-HCl pH 7.4, 25 mM sucrose and 1 mM EDTA). After centrifugation at 500–1000 *g*, the supernatant was layered over an 8 ml sucrose cushion [10 mM Tris-HCl pH 7.4, 35% (v/v) sucrose, 1 mM EDTA] in SW28 rotor tubes and centrifuged at 30,000 *g* at 4 °C for 30 min. Membranes (at the interface) were later collected using a needle and a Hamilton syringe. The interface layer was diluted with buffer 3 (10 mM Tris-HCl pH 7.4 and 0.25 mM sucrose) and membranes collected as a pellet by centrifugation for 45 min. at 100,000 *g* at 4 °C. Membranes were resuspended in 1 ml buffer 3 and stored at −80 °C. All buffers contained a cocktail of protease inhibitors (benzamidine, EDTA, aprotinin and leupeptin). Membranes from B-30 CHO cells or non-infected Sf9 cells were similarly prepared.

ATPase assay

Pgp was assayed for ATPase activity by measuring the release of inorganic P_i, essentially as described by Chifflet et al. [29], in the presence or absence of 10 μM verapamil. A P_i standard curve was prepared for each assay. All assays contained sodium azide (5 mM), EGTA (2 mM) and ouabain (1 mM). Incubation was at 37 °C for 25 min in a total reaction volume of 150 μl. Absorbance was measured at 850 nm.

Electropheresis and immunodetection

Membrane proteins were resolved on 8% Laemmli-type denaturing SDS-PAGE gels and transferred to nitrocellulose sheets for immunodetection of Pgp. Typically, 20 μg of membranes in sample buffer were loaded onto gels and electroblotted onto nitrocellulose sheets. Immunodetection was performed with C219 anti-Pgp mAb followed by a horseradish peroxidase conjugated secondary antibody. Reactive bands were visualised by detection with ECL chemiluminescent substrate (Amersham) and exposure to X-ray film. Protein concentrations were determined using the BCA kit.

Measuring ^{125}Iodide efflux

Efflux was measured using previously described techniques [30, 31]. ^{125}I efflux was used to assess Cl^- permeability in cells grown to 75–90% confluence on 22-mm plates (Costar). Cells were incubated with 10 mCi/ml ^{125}I in extracellular buffer solution containing 140 mM NaCl, 4 mM KCl, 1 mM KH_2PO_4, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 3 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH) (290 mOsm/Kg H_2O by a freezing point osmometer) at room temperature. After 1 h, cells were washed three times with normal extracellular buffer to remove extracellular isotope. Studies were initiated by adding and removing 1 ml of extracellular buffer at 1-min intervals. Cells were exposed to the potassium channel blocker 4-aminopyridine (3 mM) during the course of the experi-

ment to eliminate any contribution of potassium permeability. After a 2-min period to establish basal efflux, hypoosmotic buffer containing 93 mM NaCl, 4 mM KCl, 1 mM KH_2PO_4, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 3 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH) (90 mOsm/Kg H_2O by a freezing point osmometer) was added. Cells exposed to phorbol esters were treated with TPA (200 nM) for 5 min prior to hypoosmotic stress and during exposure to hypoosmotic stress. At the end of the study, cells were lysed with 1 ml of 0.1 M NaOH to determine the total amount of radioactivity remaining. Results were normalised by dividing the counts at each time point by the total number of remaining counts. Some of the data were presented as percentage change in peak ^{125}I efflux defined by the following equation to determine the effects of test agents on hypoosmotically stimulated efflux:

$$\%\ \text{change in peak efflux} = [(\text{peak efflux} - \text{control efflux})/\text{control efflux}] \times 100$$

Results

We have constructed a baculovirus (AcMNPV/his-MDR1) that expresses the human MDR1 protein (Pgp) in Sf9 cells. Membrane preparations from Sf9 cells infected with the recombinant baculovirus showed the presence of Pgp in the membrane fraction, as evidenced by immunostaining of western blots using the monoclonal antibody C219, which is reactive with human Pgp (Fig. 1). The expression level was lower than in CHO B-30 cell membranes, but similar to that previously reported for Sf9 cells [26, 32, 33]. Moreover, co-infection of Sf9 cells with both AcMNPV/hisMDR1 and AcMNPV/PKC(α or ε) demonstrated that both Pgp and PKC (α or ε) were present in the membrane fraction, as evidenced by double immunostaining with C219 and anti-PKC (α or ε) polyclonal antibodies (not shown). The expressed Pgp showed verapamil-stimulated ATPase activity (Fig. 2), which was similar to that previously reported for a baculovirus expression system [26].

In agreement with previous reports [26], our results demonstrate that co-expression of Pgp with PKC α elevates the ATPase activity of Pgp (Fig. 2), most likely through phosphorylation of the protein in the linker region. This is supported by studies demonstrating that co-expression of Pgp with PKC in Sf9 cells leads to Pgp phosphorylation [26]. We do not expect the simple glycosylation of Pgp in Sf9 cells to interfere with its utilisation by PKC for phosphorylation. The stimulation of the ATPase activity of phosphorylated Pgp occurred both in the presence and absence of verapamil. However, no such stimulation of activity was observed when Pgp was co-expressed with PKC ε (Fig. 2). To our knowledge this is the first report that a PKC calcium independent isoform does not affect the ATPase activity of Pgp. This may be due to phosphorylation of Pgp by this calcium independent PKC isoform at site(s) other than that responsible for stimulating the ATPase activity of Pgp (ser^{671}). Previous reports have shown that the MDR phenotype in human breast cancer cells is associated with a significant decrease in PKC ε and δ levels and a concomitant increase in PKC α levels. Taken together, this may suggest that the calcium independent PKC isoforms are not required for regulating the ATPase activity of P-glycoprotein and may not regulate drug binding/transport.

Regulation of the ATPase activity with PKC may also influence the volume-sensitive anion permeability. This was directly examined by measuring fluctuations in ^{125}I efflux, a marker for chloride permeability, in Sf9 cells expressing Pgp as well as co-expressing Pgp/PKC (α or ε). Exposure to hypoosmotic solution increased peak ^{125}I efflux over basal levels by 161 ± 7% ($n = 9$, $p < 0.05$) and 168 ± 9% ($n = 9$, $p < 0.05$) for Pgp/

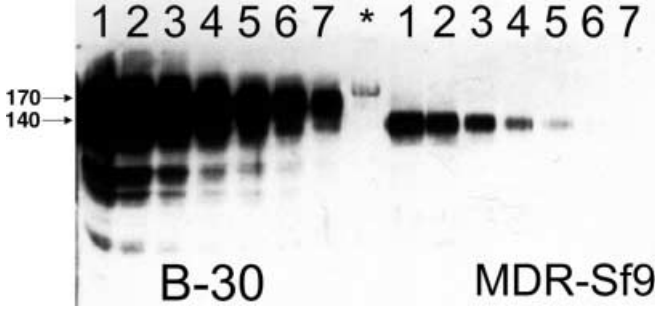

Fig. 1 Immunodetection of baculovirus expressed Pgp. Membrane preparations from Sf9 cells expressing human Pgp (MDR-Sf9) were fractionated on 8% SDS-PAGE, transferred to nitrocellulose and immunostained with the mAb C219/HRP secondary Ab using the ECL detection system. Membranes from CHO B-30 cells (B-30) were also immunostained as control. *Lanes 1–7* [B-30 and MDR-Sf9 membrane aliquots (≈140–20 μg total protein, at ≈20 μg increments), were loaded in *lanes 1–7*, respectively]. *Marker lane* (*) denotes myosin (MW 220 kDa). Pgp from B-30 and Sf9 migrates at about 170 and 140 kDa, respectively (*arrows*)

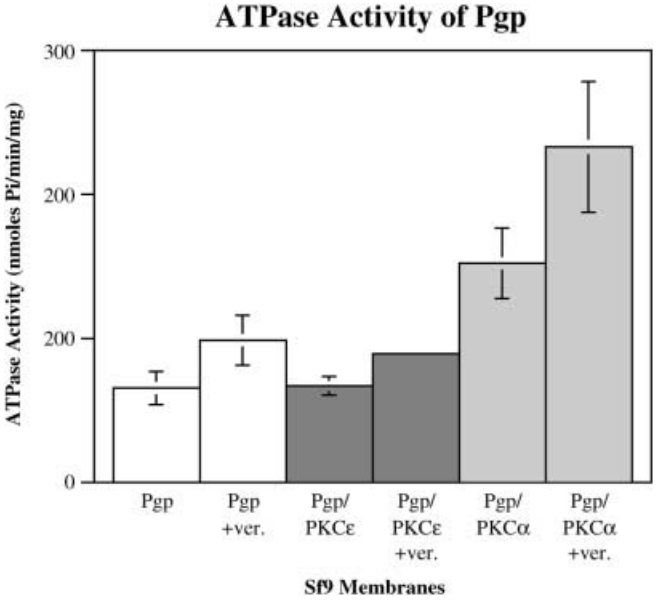

Fig. 2 ATPase activity of baculovirus expressed Pgp with or without membrane PKC. Membrane preparations from Sf9 cells expressing human Pgp or co-expressing Pgp and PKC α or PKC ε. The ATPase activity was determined in the presence or absence of 10 μM verapamil (*ver*). The data is the average of three separate measurements (± SE)

PKC α and Pgp/PKC ε expressing cells, respectively. Stimulation of swelling-activated ^{125}I efflux was observed within 1-min exposure to hypoosmotic solution and approached a maximal stimulation after 2 min. Such enhanced efflux most likely represents activation of latent endogenous Sf9 Cl^- volume-sensitive Channel(s). This increase was followed by a gradual decline in basal levels over the next 2 min, which may represent RVD. No significant difference in the hypoosmotic response discussed above was observed between infected and control Sf9 cells (Fig. 3). There was no significant difference in hypotonic-regulated iodide efflux between control and Pgp expressing cells.

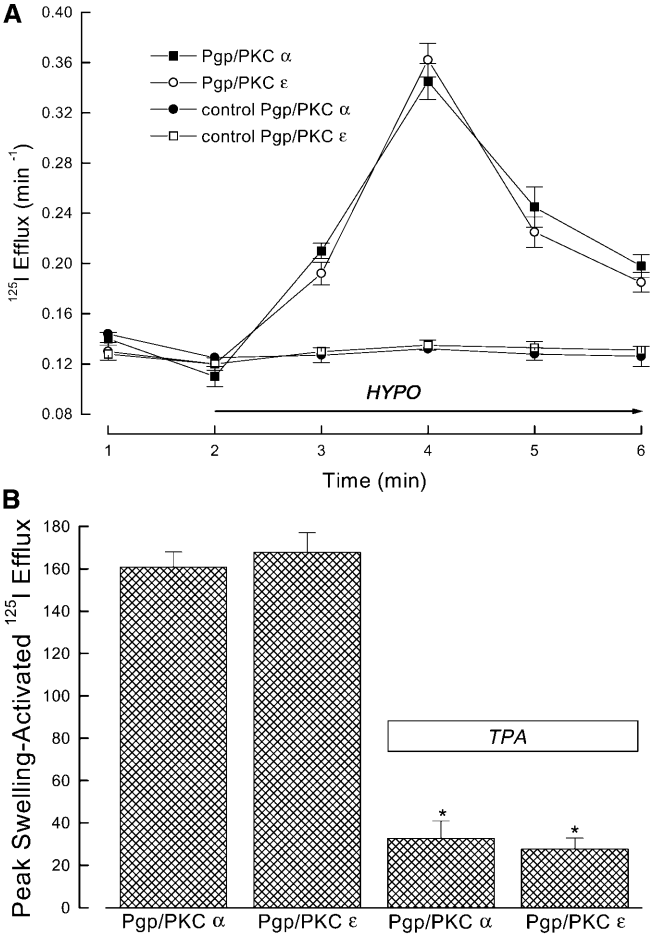

Fig. 3A,B Effects of Pgp/PKC expression on swelling-activated ^{125}I efflux. Recombinant baculovirus was used to co-express Pgp/PKC α or Pgp/PKC ε in Sf9 cells. (**A**) Exposure to hypoosmotic solution rapidly increased ^{125}I efflux in cells expressing either isoform of PKC [Pgp/PKC α (■); Pgp/PKC ε (○)]. Under control conditions (non-exposure to hypotonic solution), no significant alterations in ^{125}I efflux were observed with either isoform of PKC [Pgp/PKC α (●); Pgp/PKC ε (□)]. (**B**) Cells pre-treated with TPA (200 nM) for 15 min prior to hypoosmotic solution, and in the continued presence of TPA during exposure to hypoosmotic stress, significantly ($^*P < 0.05$) inhibited the swelling-activated ^{125}I efflux. Similar inhibitory effects were observed with both isoforms of PKC expression. *Error bars* (SE) are not evident when they are smaller than the size of the symbol

The effects of 'maximal' phosphorylation of Pgp by either PKC α or PKC ε on the swelling-activated ^{125}I efflux were investigated by pre-treating the cells for 15 min with TPA (200 nM) which stimulates PKC activity. After obtaining basal ^{125}I efflux, the cells were exposed to hypoosmotic solution. In the continued presence of TPA, hypoosmotic stress inhibited the swelling-activated ^{125}I efflux by 67% ± 8% ($n = 6$) and 72% ± 5% ($n = 6$) for PKC α and for PKC ε, respectively, when compared with non-treated cells.

Discussion

This study examined the effect(s) of co-expressing Pgp with two PKC isoforms (α and ε) in Sf9 insect cells on the ATPase activity of Pgp and Sf9 cell anion permeability. The baculovirus expression system is ideal for such studies as it allows for post-translational modification of the expressed protein and for the selective introduction of various PKC isoforms that are normally absent from Sf9 cells.

Our results with PKC α are in closer agreement with the results of Ahmad et al. [26] than those of Szabo et al. [32]. These latter workers reported only very modest stimulation of the ATPase activity when they mimicked the charge effect of phosphorylation through three Ser-Glu substitutions in the linker region of Pgp. However, such mutations may have caused a conformational change in the mutant Pgp, thereby reducing the level of stimulation of the ATPase activity through the 'mimicked' phosphorylation charge. Moreover, it is not clear whether PKC α phosphorylates all three serines (661, 667 and 671) in Sf9 cells. It is possible that phosphorylation of one serine stimulates Pgp activity, whilst phosphorylation of two or three negates this stimulation. The study of Szabo et al. substituted all three serines (661, 667 and 671) simultaneously, hence it was difficult to infer if a single substitution (e.g. ser^{671}–glu^{671}) would have caused a greater stimulation of the ATPase activity. It is noteworthy that PKC can phosphorylate three serines in the linker region of Pgp, yet mutating only one serine (ser^{671}) to asparagine is sufficient to nullify such stimulation of the ATPase activity of Pgp [26].

Results from our efflux studies are similar to the observations by Hardy et al. [10] in HeLa cells and NIH3T3 cells expressing Pgp, where stimulation of PKC by TPA reduced $I_{Cl,swell}$. In contrast, Bond et al. [35] reported that a similar pre-treatment with TPA did not significantly alter $I_{Cl,swell}$ in CHO cells (LR73 cells) expressing mouse Pgp1a or Pgp1b. Bond et al. [35] further suggested that these differences in $I_{Cl,swell}$ activation with TPA pre-treatment may correlate with the differing intracellular concentrations of PKC among various cell types, since the endogenous levels of PKC in LR73 cells were found to be lower. Additional experiments confirmed the authors' assumption, as the inclusion of PKC in the pipette solution in TPA pre-treated LR73 cells reduced whole-cell $I_{Cl,swell.}$ However, the different PKC

isoforms were not examined. In the present study no significant differences were observed between cells transfected with Pgp/PKC α or Pgp/PKC ε in the absence of TPA. However, PKC α expressing cells showed a lower rate of initial efflux as compared with PKC ε expressing and control cells. The swelling-activated ^{125}I efflux was significantly inhibited with TPA pre-treatment in Sf9 cells co-expressing Pgp/PKC (α or ε). Since TPA-activates PKC, this may suggest that PKC hyper-phosphorylation of Pgp acts as a signal to prevent activation of $I_{Cl,swell}$. Our results with PKC α and PKC hyper-suggest that regulation of Pgp's ATPase and volume-sensitive anion efflux activities can be independent of one another.

The role of phosphorylation in activating the volume-sensitive anion channel has been controversial. Meyer and Korbmacher [36] in cultured mouse collecting duct cells and Carpenter and Peers [37] in carotid body type I cells suggested that phosphorylation of the volume-sensitive anion channel and/or accessory proteins is required for activation. Furthermore, Kartner et al. [38] demonstrated that phosphorylation regulates anion conductance in Sf9 cells expressing CFTR. In contrast, other investigators have reported that non-hydrolysable ATP analogues caused normal activation of the volume-sensitive anion channel, in the presence or absence of intracellular Mg^{2+} [reviewed by 39, 40]. This indicates that phosphorylation of the channel or accessory proteins are not required for activation of $I_{Cl,swell}$ in these cells. Furthermore, phosphorylation of Pgp by PKC can cause a decrease in $I_{Cl,swell}$ in some cell types, while no significant effects were observed in others. Additionally, Vanoye et al. [41] suggested that in Pgp expressing cells, PKC activation reduces the rate of increase in $I_{Cl,swell}$, whilst PKA activation reduces $I_{Cl,swell}$, without affecting the rate of increase; the effect of both kinases on $I_{Cl,swell}$ was also additive. Interestingly, other studies reported no effect of TPA on $I_{Cl,swell}$ in cells expressing Pgp or ATPase defective Pgp [13].

In summary our results demonstrate that PKC α, but not PKC ε, stimulates the ATPase activity of Pgp. Additionally, TPA activation of PKC α and PKC ε leads to inhibition of the volume-sensitive anion efflux. The effect of PKC α on the ATPase activity of Pgp probably reflects the 'minimal' level of phosphorylation (e.g. of ser^{671}) which is required to stimulate the ATPase activity, but insufficient (unless augmented through the action of TPA) to influence $I_{Cl,swell}$. PKCα possibly phosphorylates more residues in Pgp (thereby affecting both ATPase activity and $I_{Cl,swell}$) than PKC ε, which may be restricted to phosphorylating only those residues necessary for regulating $I_{Cl,swell}$ (perhaps ser^{667}/ser^{667}). Whilst this report was in preparation, Sachs et al. reported that PKC α and PKC ε differentially phosphorylate Pgp's linker region [43], supporting the argument that different isoforms phosphorylate different residues in the linker region. Such selective phosphorylation by the PKC isoforms may confer finer regulation of Pgp's functions. Hierarchical multiple phosphorylation of Pgp may act to shuttle the protein between its ATPase driven drug transport and swelling activated channel regulation states [44]. Drug efflux and cell swelling have previously been shown to be mutually exclusive [42]. Whether Pgp phosphorylation with other kinases (e.g. CKII and Tyr kinases) also regulates $I_{Cl,swell}$ remains to be determined. The baculovirus expression system could be utilised to examine regulation of Pgp activities with other PKC isoform and/or protein phosphatases (e.g PP1/PP2A). It is suitable for expression of correctly folded and functional ion channels and channel regulatory proteins [44].

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