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Protein kinase C-mediated phosphorylation of the human multidrug resistance P-glycoprotein regulates cell volume-activated chloride channels

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The multidrug resistance P-glycoprotein (P-gp), which transports hydrophobic drugs out of cells, is also associated with volume-activated chloride currents. It is not yet clear whether P-gp is a channel itself, or whether it is a channel regulator. Activation of chloride currents by hypotonicity in cells expressing P-gp was shown to be regulated by protein kinase C (PKC). HeLa cells exhibited volume-activated chloride currents indistinguishable from those obtained in P-gp-expressing cells except that they were insensitive to PKC. HeLa cells did not express detectable P-gp but, following transient transfection with cDNA encoding P-gp, the volume-activated channels acquired PKC regulation. PKC regulation was abolished when serine/threonine residues in the consensus phosphorylation sites of the linker region of P-gp were replaced with alanine. Replacement of these residues with glutamate, in order to mimic the charge of the phosphorylated protein, also mimicked the effects of PKC on channel activation. These data demonstrate that PKC-mediated phosphorylation of P-gp regulates the activity of an endogenous chloride channel and thus indicate that P-gp is a channel regulator.

Key words: chloride channels/multidrug resistance/P-glycoprotein/protein kinase C/phosphorylation/volume regulation

Introduction

Expression of the human multidrug resistance P-glycoprotein (P-gp) can confer resistance of cells to chemotherapeutic drugs (Gottesman and Pastan, 1993). P-gp is a member of the ABC superfamily of transporters (Higgins, 1992) and utilizes ATP to pump hydrophobic drugs out of cells, decreasing their intracellular concentrations and hence their toxicity. In addition to its function as a drug transporter we and others have shown that increased P-gp expression in several cell types is associated with characteristic chloride currents which are activated in response to cell swelling (Gill *et al.*, 1992; Valverde *et al.*, 1992; Altenberg *et al.*, 1994; Luckie *et al.*, 1994). The drug transport and chloride channel activities associated with P-gp are distinct and can be separated by their requirements for ATP hydrolysis (Gill *et al.* 1992) and by their pharmacology (Mintenig *et al.*, 1993). The simplest interpretation of these results is that P-gp is itself a chloride channel, or a component thereof, and we originally favoured this hypothesis because the related cystic fibrosis protein, CFTR, had been shown to be a chloride channel (Anderson *et al.*, 1991; Bear *et al.*, 1992). However, these data (Gill *et al.*, 1992; Valverde *et al.*, 1992) do not exclude the alternative hypothesis that P-gp up-regulates an endogenous volume-activated chloride channel. In addition, recent findings that the level of P-gp expression in certain cell types does not correlate with the magnitude of cell volume-regulated chloride currents imply a complex relationship between P-gp and channel activity (Luckie *et al.*, 1994; Rasola *et al.*, 1994). We have therefore investigated the regulation of P-gp-associated chloride channels.

Drug transport by P-gp is modulated by protein-kinase C (PKC)-mediated phosphorylation, although the mechanisms are still obscure. Overexpression of PKC, or treatment with compounds which activate PKC, can enhance drug resistance or drug efflux (Fine *et al.*, 1988; O'Brian *et al.*, 1989; Chambers *et al.*, 1990; Ma *et al.*, 1991; Yu *et al.*, 1991; Bates *et al.*, 1992). There are a number of consensus PKC phosphorylation sites in the linker region of P-gp which separates the two halves of the molecule and at least some of these sites are phosphorylated *in vivo* and *in vitro* (Chambers *et al.*, 1993; Orr *et al.*, 1993). We therefore examined whether PKC might also influence the chloride channel activity associated with P-gp. Activation of endogenous chloride channels was shown to be regulated by PKC-mediated phosphorylation of P-gp. This is evidence of a functional role for P-gp phosphorylation. In addition, the data indicate that P-gp is a regulator of an endogenous channel rather than possessing intrinsic channel activity.

Results

TPA inhibits volume-activated chloride currents associated with P-gp expression

We have previously characterized volume-regulated chloride currents in NIH-3T3*MDR1* cells which overexpress P-gp (Gill *et al.*, 1992; Valverde *et al.*, 1992). In order to examine whether PKC plays a role in the regulation of these currents, the effects of the PKC activator phorbol 12-myristate 13-acetate (TPA: Nishizuka, 1986; Hug and Sarre, 1993) were studied. Figure 1A shows a recording for a single NIH-3T3*MDR1* cell. When the cell was bathed in isotonic medium, negligible chloride currents were observed in whole-cell patch clamp recordings in response to voltage pulses between −80 and +80 mV (panel a). On exposure to hypotonic bathing solution, characteristic outwardly rectifying currents appeared

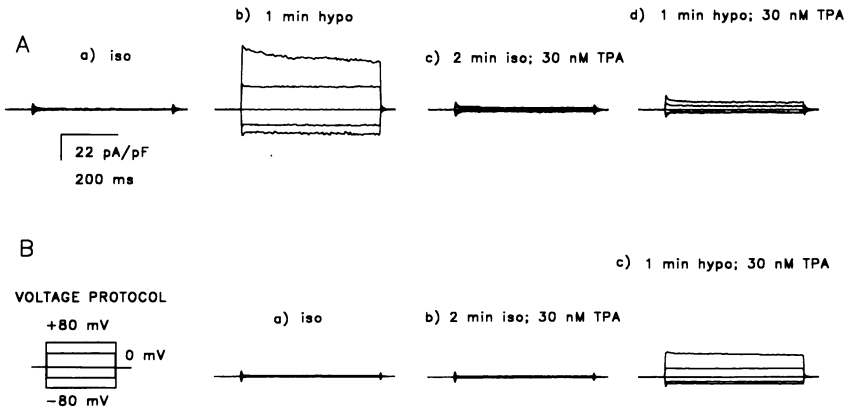

Fig. 1. P-gp-associated chloride currents are sensitive to TPA. Representative whole-cell patch-clamp recordings for single cells are shown. Mean data for many independent cells are shown in Figure 2A. Cells were held at 0 mV and pulsed from −80 to +80 mV in 40 mV steps. The voltage protocol is shown in (**B**). The currents were normalized for cell size (capacitance). (**A**) An NIH-3T3*MDR1* cell was exposed to isotonic conditions and chloride currents recorded. No significant currents could be detected when the cells were left for several minutes in isotonic conditions (panel a). The cell was then exposed to a hypotonic bathing medium and currents recorded after 1 min; sizeable, outwardly rectifying currents were observed (panel b). The cell was then returned to isotonic conditions. In the presence of 30 nM TPA (panel c) and following exposure to hypotonicity in the continued presence of TPA (panel d), no significant increase in currents were observed. Thus, hypotonicity failed to elicit chloride currents in the presence of TPA. (B) A parental NIH-3T3 fibroblast was exposed to isotonic conditions and chloride currents were recorded (panel a). The cell was then transferred to isotonic medium containing 30 nM TPA and currents were recorded after 2 min (panel b). The cell was then transferred to hypotonic medium in the continued presence of TPA and currents recorded after 1 min; small chloride currents were elicited (panel c), indistinguishable from those elicited in the same cells in the absence of TPA.

within 1–2 min (panel b) and these were reversed when the cell was returned to isotonic bathing conditions (panel c). However, after exposure to 30 nM TPA for 2 min, currents could not be elicited by hypotonicity (panel d). The mean values obtained for similar experiments carried out on 22 independent cells are shown in Figure 2A. Clearly, pre-treatment with TPA prevents channel activation. As PKC can also be activated by high intracellular Ca^{2+} concentrations (Hug and Sarre, 1993), we examined the effects of Ca^{2+} on channel activation. Chloride currents in NIH-3T3*MDR1* cells could not be activated when cells were dialysed with 1.7 μM free calcium in the pipette solution (Figure 2A). These data show that factors which activate PKC prevent the development of volume-activated chloride currents in these P-gp-expressing cells.

To ascertain whether the sensitivity of these chloride currents to PKC activation was P-gp dependent, the parental NIH-3T3 cells were studied. Figure 1B shows a representative recording obtained for a single NIH-3T3 cell. As expected, under isotonic conditions negligible chloride currents were detected (panel a) and these were unaffected by 30 nM TPA (panel b). Following exposure of the cell to a hypotonic bathing solution, chloride currents were activated. The currents in NIH-3T3 cells had similar electrophysiological characteristics to those obtained for NIH-3T3*MDR1* cells but were of significantly smaller magnitude, as reported previously (e.g. Valverde *et al.*, 1992, Figure 1). The mean current after 1 min hypotonicity in the presence of TPA was not significantly different from currents recorded in the same cells in the absence of TPA (14 ± 4 pA/pF; n = 10). Thus, in contrast to the NIH-3T3*MDR1* cells, the development of volume-activated chloride currents in NIH-3T3 cells was unaffected by exposure to TPA. These results suggest that the sensitivity of channel activation to TPA depends upon expression of P-gp.

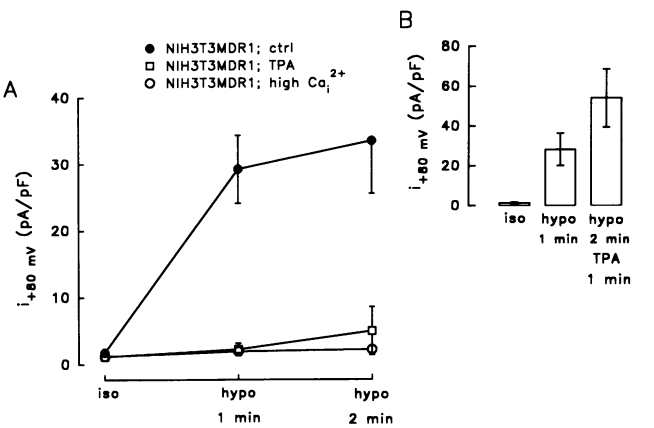

Fig. 2. Effect of TPA and calcium on volume-activated currents in NIH-3T3*MDR1* cells. (**A**) Mean currents at +80 mV were measured in isotonic conditions (iso) and after 1 or 2 min in hypotonic bathing solution (hypo 1 min and hypo 2 min, respectively). ● cells dialysed with standard intracellular pipette solution containing 150 nM free Ca^{2+} (n = 22); ○ cells dialysed with 1.7 μM free Ca^{2+} in the pipette solution (n = 5). □ cells dialysed with standard intracellular pipette solution but exposed to 30 nM extracellular TPA (n = 5). No significant currents were detected if the cells were retained in isotonic conditions (data not shown). (**B**) Mean currents obtained in NIH-3T3*MDR1* fibroblasts under isotonic conditions (iso), after 1 min in hypotonicity (hypo 1 min) and after a further 1 min exposure to 30 nM TPA (hypo 2 min TPA 1 min; n = 4). The addition of TPA did not reverse currents pre-activated by hypotonicity.

TPA does not block activated channels

A number of hydrophobic compounds block the volume-activated currents in cells expressing P-gp (Valverde *et al.*, 1992; Mintenig *et al.*, 1993). To exclude the possibility that the effects of TPA were simply due to blockade of the activated channel, currents in NIH-3T3*MDR1* cells were induced by hypotonicity and, after 1 min incubation in hypotonic medium, TPA was added to the bath solution.

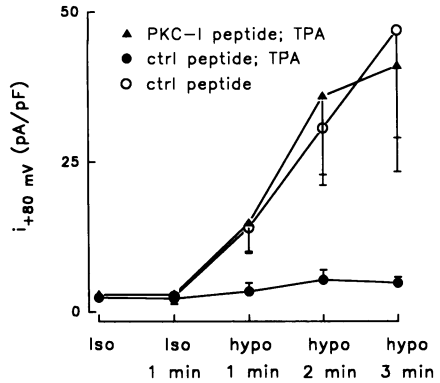

Fig. 3. Effect of PKC pseudosubstrate inhibitors on volume-activated chloride currents in NIH-3T3*MDR1* cells. Where appropriate, TPA was added to the bath solution at the start of the experiment. Mean currents were measured at +80 mV under isotonic conditions at time 0 (iso), after 1 min in isotonic conditions (iso 1 min) and after 1, 2 or 3 min in a hypotonic bathing solution (hypo). ○ cells dialysed against the control peptide (1 μM); large currents developed on exposure to hypotonicity (n = 14). ● cells dialysed against the control peptide with TPA (30 nM) included in the bath solution; exposure to TPA prevented channel activation (n = 10). ▲ cells dialysed against the PKC pseudosubstrate inhibitory peptide (1 μM) with TPA (30 nM) included in the bath solution (n = 11); TPA did not prevent channel activation in the presence of the pseudosubstrate peptide.

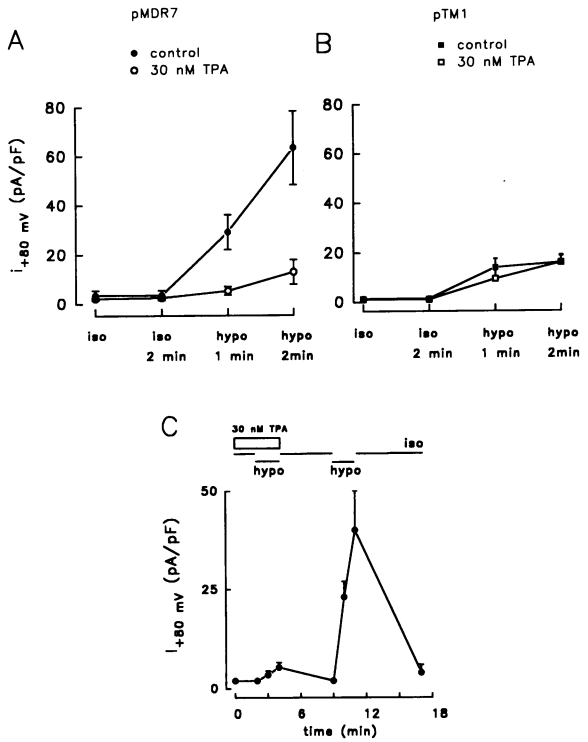

Fig. 4. Effect of TPA on currents generated by transient expression of P-gp in NIH-3T3 fibroblasts. (**A**) Mean whole cell currents recorded in NIH-3T3 fibroblasts transiently transfected with the P-gp expression plasmid pMDR7. Currents were measured in isotonic conditions (iso) and 1 and 2 min after transfer to hypotonic conditions (hypo). ● currents obtained in response to hyptonicity (n = 18). ○ currents obtained in response to hypotonicity in the presence of 30 nM TPA (n = 12). (**B**) Mean whole cell currents obtained in NIH-3T3 fibroblasts transiently transfected with the vector pTM1. ■ currents obtained in response to hypotonicity (n = 8); □ currents obtained in response to hypotonicity in the presence of 30 nM TPA (n = 5). (**C**) Individual cells, transiently transfected with the P-gp expression plasmid pMDR7, were monitored under alternating isotonic and hypotonic conditions in the presence and then absence of 30 nM TPA (n = 10). The experimental protocol to which each cell was subjected is indicated above the graph.

The magnitude of the chloride currents in the presence of TPA (Figure 2B) was not significantly different from those in the absence of TPA (Figure 2A). Thus 30 nM TPA does not block the activated channels and must therefore reduce currents by inhibiting channel activation.

The effects of TPA are mediated via PKC

To demonstrate that the effects of TPA on channel activation are mediated via PKC, a pseudosubstrate peptide inhibitor of PKC was used (House and Kemp, 1987; Kemp *et al.* 1991). This peptide is efficiently phosphorylated by PKC and titrates out PKC activity in the cell. As a control, we used a peptide identical to the pseudosubstrate peptide, except that the phosphorylated serine was replaced by an alanine; this peptide cannot be phosphorylated and hence does not interfere with PKC activity. The data in Figure 3 show that when the PKC inhibitor peptide was included in the pipette solution at a concentration of 1 μM, TPA no longer prevented channel activation. In contrast, when similar concentrations of the control peptide were included in the pipette solution, TPA prevented activation of the chloride currents. The control peptide alone did not modify the response to hypotonicity. These data demonstrate that the effects of TPA on channel activity are mediated via the activation of PKC.

The effect of PKC on volume-activated chloride currents requires P-gp expression

The above data show that TPA inhibits channel activation in NIH-3T3*MDR1* cells but not in the parental NIH-3T3 cells. This suggests that P-gp expression is required for PKC to influence channel activity. The phenotype of permanently transfected cell lines may diverge from that of the parent cell line after many passages in cell culture, and an unequivocal way of demonstrating that P-gp is responsible for the observed differences in channel regulation by PKC was obtained using a transient transfection system (Figure 4). This excludes the possibility of changes unrelated to P-gp and also avoids the requirement for a selective drug in the culture of NIH-3T3*MDR1* cells. Transfection of NIH-3T3 cells with the P-gp expression plasmid pMDR7 generated a considerable increase in volume-activated chloride currents (Figure 4A), while transfection with the vector pTM1 (Figure 4B) had no significant effect on the magnitude of the currents. As the only difference between cells transiently transfected with pMDR7 and pTM1 is expression of P-gp, these results show that increased expression of P-gp increases the magnitude of the currents. These data are similar to those reported previously (Gill *et al.*, 1992; Valverde *et al.*, 1992). Next, the effect of TPA on these currents was studied. In NIH-3T3 cells transfected with the vector pTM1, TPA had no effect on the response to hypotonicity (Figure 4B). However, when the cells were transfected with pMDR7, exposure to 30 nM TPA inhibited activation of chloride currents (Figure 4A). This latter result is shown in more detail in Figure 4C, which summarizes the responses of 10 independent cells transfected with pMDR7. The response to hypotonicity was examined both in the

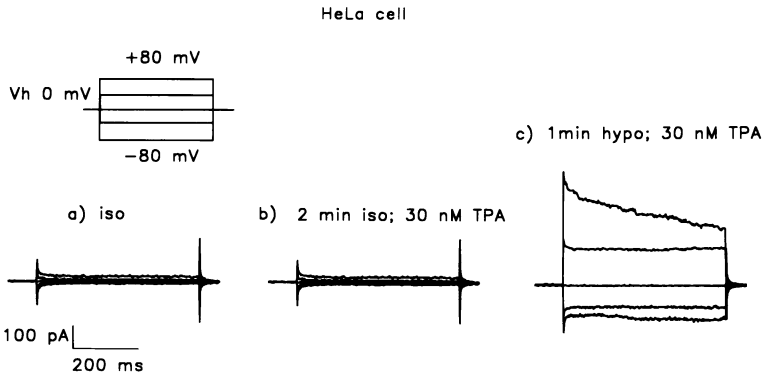

Fig. 5. TPA does not inhibit volume-activated chloride currents in HeLa cells. The figure shows the voltage protocol used and representative whole cell recordings for a single HeLa cell. Negligible currents were recorded in isotonic bathing solution (panel a) or after 2 min in isotonic solution containing 30 nM TPA (panel b). When the cell was exposed to a hypotonic bathing solution containing 30 nM TPA, sizeable outwardly rectifying currents were recorded (panel c). The capacitance of the cell recorded was 25 pF.

presence and absence of TPA. In the presence of TPA, only very small chloride currents could be induced by hypotonicity. When the TPA was removed, large currents could be induced by hypotonicity, demonstrating that functional volume-activated channels were present in the cell but that their activation was impaired by TPA. In total, these data show that P-gp expression confers PKC sensitivity upon the volume-regulated chloride currents in NIH-3T3 cells.

Volume-activated chloride channels in HeLa cells

On exposure to hypotonicity, HeLa cells develop large, volume-activated chloride currents which are indistinguishable from those seen in NIH-3T3*MDR1* cells, in terms of ion selectivity, time-dependent inactivation at depolarizing potentials and pharmacology (Diaz *et al.*, 1993). However, HeLa cells do not display a multidrug resistance phenotype and P-gp expression could not be detected by Western blotting (Figure 6). This suggested that P-gp is unlikely to be the channel protein responsible for the endogenous chloride currents of HeLa cells.

Unlike the volume-activated chloride currents in P-gp-expressing NIH-3T3*MDR1* cells, activation of the currents in HeLa cells was not inhibited by TPA (Figure 5), or by increased pipette (intracellular) concentrations of free calcium (1.7 μM; $n = 5$; data not shown). To determine whether P-gp can regulate these endogenous chloride channels, HeLa cells were transiently transfected with the P-gp expression plasmid pMDR7. Expression of P-gp was confirmed by Western blotting (Figure 6). Figure 7A shows results obtained for a single pMDR7-transfected HeLa cell. Expression of P-gp conferred TPA sensitivity on the endogenous channels (Figure 7A; panels a–c). This inhibition was reversible as, after 5 min recovery in isotonic conditions in the absence of TPA, exposure to hypotonicity resulted in large outwardly-rectifying chloride currents (Figure 7A; panels d and e). Similar results were obtained for 10 independent cells (Figure 7B). Figure 8 summarizes the mean results obtained for similar experiments on cells challenged with hypotonic solutions in the presence or absence of TPA. Transfection with pMDR7, but not the control vector pTM1, conferred TPA sensitivity on the endogenous chloride currents of HeLa cells. It should be noted that the magnitude of the chloride currents evoked by hypotonicity was not influenced by transfection with either pMDR7 or the vector pTM1. (Volume-activated chloride currents in untransfected HeLa cells were 20 ± 15 pA/pF after 1 min and 38 ± 10 pA/pF after 2 min; $n = 4$.) Thus, expression of P-gp confers PKC sensitivity on the endogenous chloride currents of HeLa cells. These results strongly suggest that, at least in HeLa cells, P-gp is not the channel protein itself, but that it acts as a regulator of an endogenous channel protein.

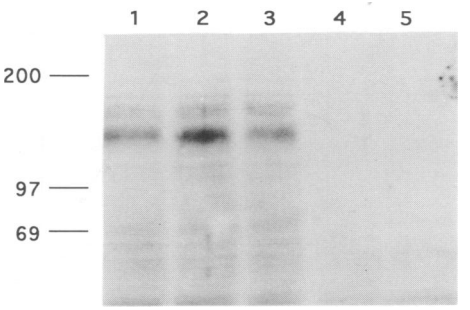

Fig. 6. Western blot showing expression of P-gp following transfection of HeLa cells. Twenty μg protein was loaded into each lane. Lane 1, HeLa cells transfected with the P-gp expression plasmid pMDR7; lane 2, HeLa cells transfected with pMDR737 expressing the mutant P-gp8A; lane 3, HeLa cells transfected with pMDR738 expressing the mutant P-gp8E; lane 4, HeLa cells transfected with the vector pTM1; lane 5, untransfected HeLa cells. Molecular weight markers are indicated.

Mutation of the consensus PKC phosphorylation sites of P-gp

To demonstrate that the PKC-mediated regulation of chloride channels occurrs via the phosphorylation of P-gp, the consensus phosphorylation sites of P-gp were altered by site-directed mutagenesis. The linker region of P-gp contains the major sites of PKC-mediated phosphorylation, both *in vivo* and *in vitro* (Chambers *et al.*, 1993; Orr *et al.*, 1993). There are eight serine/threonine residues in the 'linker' region which correspond to consensus PKC phosphorylation sites (Figure 9A). As it is not known which, if any, of these sites is functionally significant, all eight residues were altered. Two mutants were constructed. In one, the serine/threonine residues were replaced with alanine (P-gp8A) in order to prevent phosphorylation and

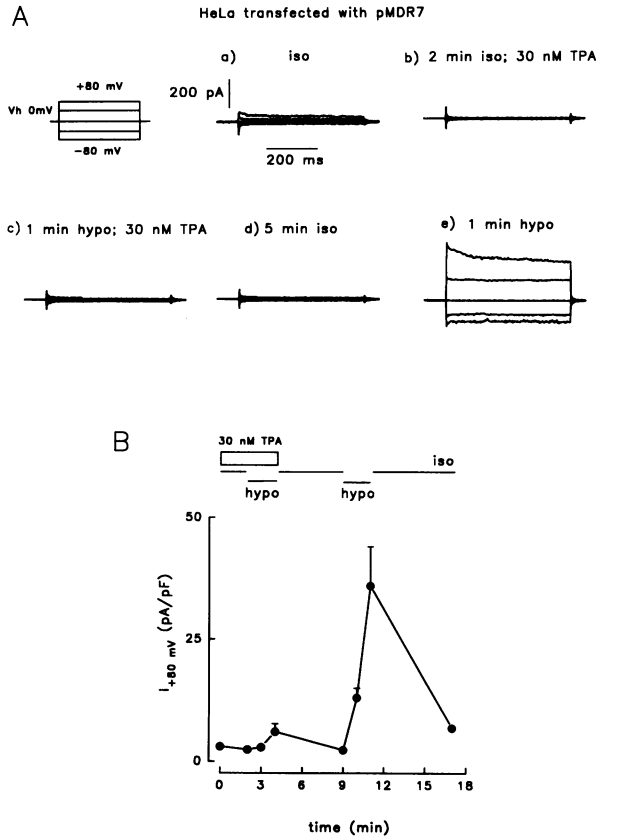

Fig. 7. Effect of TPA on HeLa cells transfected with the P-gp expression plasmid pMDR7. **(A)** Representative whole cell recordings for a single HeLa cell transiently transfected with pMDR7. The voltage protocol is shown. No currents were elicited in isotonic conditions in the absence of TPA (panel a) or 2 min after adding 30 nM TPA to the bathing solution (panel b). The cell was then exposed to a hypotonic bathing solution containing TPA (panel c) and subsequently to an isotonic bathing solution with TPA omitted (panel d). Finally, the cell was exposed to a hypotonic bathing solution with no TPA (panel e). The capacitance of the cell recorded was 19 pF. **(B)** Individual HeLa cells transiently transfected with pMDR7 were challenged with hypotonic and isotonic bathing solutions in the presence and absence of 30 nM TPA. Mean currents (±SEM) are given for 10 independent cells. The experimental protocol to which each cell was subjected is indicated above the graph.

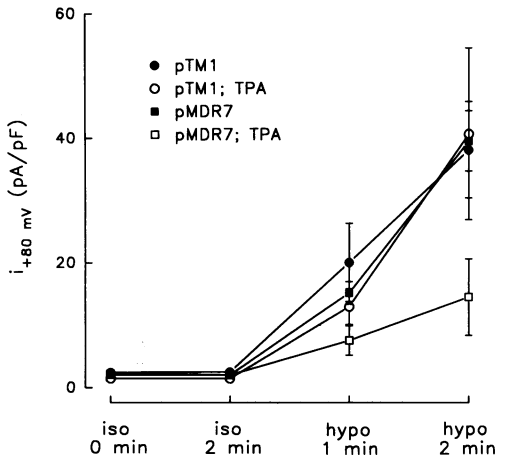

Fig. 8. Effect of TPA on volume-activated chloride currents in HeLa cells transfected with pMDR7. Mean currents were recorded for each cell at +80 mV under isotonic conditions (iso) and after 1 or 2 min in hypotonic conditions (hypo). ■ HeLa cells transiently transfected with the P-gp expression plasmid pMDR7 (n = 17); □ HeLa cells transiently transfected with pMDR7 with 30 nM TPA included in the bathing solution (n = 15); ● HeLa cells transfected with the vector pTM1 (n = 4); ○ HeLa cells transfected with the vector pTM1 with 30 M TPA included in the bathing solution (n = 8). At the 2 min time point, the currents induced in HeLa cells transfected with pMDR7 were not significantly different from those induced in cells transfected with the vector pTM1 ($P > 0.05$). Similarly there was no significant difference between the currents induced in cells transfected with pTM1 in the presence or absence of TPA ($P > 0.05$). However, a significant difference in the magnitude of currents was observed for cells transfected with pMDR7 in the presence or absence of TPA ($P = 0.003$).

mimic the non-phosphorylated state of P-gp. In the other (P-gp8E), the same residues were replaced with glutamic acid; this will also prevent phosphorylation of these sites but, in addition, mimic the negative charge of phosphorylation. Similar replacement of protein kinase phosphorylation sites with negatively charged residues in the R-domain of the cystic fibrosis chloride channel has been shown to mimic the effect of phosphorylation (Rich *et al.*, 1993).

In HeLa cells, transiently transfected with plasmid pMDR737 expressing P-gp8A (Figure 9B), the magnitude of the volume-activated chloride currents was indistinguishable from that of untransfected HeLa cells, of HeLa cells transfected with the vector pTM1 or the wild type P-gp-expression plasmid pMDR7 (compare with data in Figure 8). Western blot analysis showed the expression of the mutant protein was indistinguishable from that of the wild type (Figure 6). In contrast to cells expressing wild-type P-gp, volume-activated chloride currents in cells expressing P-gp8A were not sensitive to 30 nM TPA (Figure 9B). Thus, when P-gp was mutated such that sites in the linker region could no longer be phosphorylated, it no longer conferred TPA sensitivity on endogenous chloride channels.

Expression of P-gp8E in HeLa cells (Figure 9B) markedly reduced the activation of chloride currents compared with untransfected cells or cells expressing wild-type P-gp or P-gp8A. The magnitude of currents recorded in P-gp8E expressing cells was also not affected by exposure to TPA (Figure 9B). This result is consistent with the hypothesis that the phosphorylated state of P-gp inhibits channel activation and that the charges introduced in the P-gp8E mutant mimic this phosphorylated state.

In summary, these data demonstrate that P-gp is a channel regulator and that the effects of PKC on channel activation are mediated via the phosphorylation of P-gp.

Discussion

Human P-gp is an active transporter which pumps hydrophobic compounds out of cells. In addition to its active transport activity we and others have shown that P-gp expression is also associated with increased cell volume-activated chloride currents in certain cell types (Gill *et al.*, 1992; Valverde *et al.*, 1992; Altenberg *et al.*, 1994; Luckie *et al.*, 1994). P-gp can phosphorylated by PKC *in vitro* and *in vivo* (Chambers *at al.*, 1993; Orr *et al.*, 1993), although the functional significance of this, if any, is unclear. We have shown here that PKC-mediated phosphorylation of P-gp can regulate the activity of cell volume-activated chloride channels. In addition, these

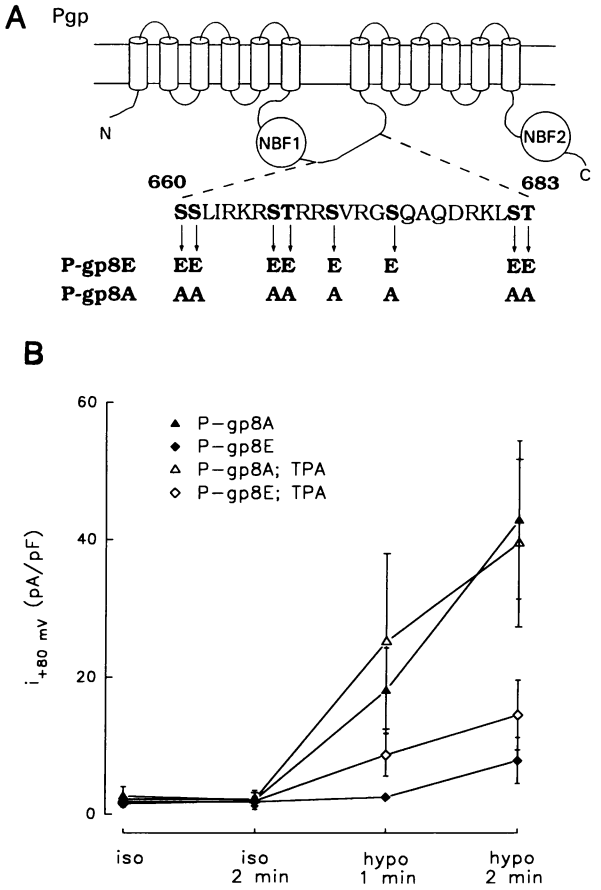

Fig. 9. Mutation of the consensus phosphorylation sites of P-gp. (**A**) Diagram of P-gp showing the two transmembrane domains and two nucleotide-binding domains (NBF1 and NBF2). The 'linker' region is located between the two halves of the molecule and part of its sequence (amino acids 660–684 of P-gp) containing the consensus phosphorylation sites is shown. The residues altered in the P-gp8E and P-gp8A mutants are shown. (**B**) Mean currents at +80 mV for HeLa cells transiently transfected with plasmids pMDR737 and pMDR738 expressing, respectively, the mutant P-gp8A and P-gp8E. Currents for each cell were measured under isotonic conditions (iso) and after 1 and 2 min in hypotonic solution. ▲ cells expressing P-gp8A (n = 6); △ cells expressing P-gp8A pretreated with TPA (n = 5); ◆ cells expressing P-gp8E (n = 8); ◇ cells expressing P-gp8E and pretreated with TPA (n = 8). After 2 min hypotonicity there was a significant difference in the magnitude of the currents induced in cells transfected with P-gp8A and P-gp8E (P = 0.006). There was statistically no significant effect of TPA on cells transfected with either P-gp8A or P-gp8E ($P > 0.05$).

studies have impinged on the question of whether P-gp is a channel or a channel regulator.

Channel regulation by PKC

The data presented here show that the P-gp-associated, cell volume-activated chloride currents are regulated by PKC. Treatment of cells with TPA prevented activation of the channels by hypotonicity but did not inhibit channels once they had been activated by hypotonicity, showing that this inhibition is not due to channel blockade. A highly specific pseudosubstrate peptide inhibitor of PKC prevented TPA inhibiting channel activation, demonstrating that the effects of TPA were mediated via PKC. Several other lines of evidence are also consistent with the effects of TPA being mediated via PKC. Short exposures (2 min) to low extracellular concentrations (30 nM) of TPA were effective and high intracellular Ca^{2+} concentrations, which also activates PKC, prevented channel activation. Furthermore the effects of TPA could be rapidly reversed.

The effects of PKC on channel activation require expression of P-gp. First, in cells not expressing human P-gp (NIH-3T3 fibroblasts and HeLa cells), the endogenous cell volume-activated chloride currents were not sensitive to TPA; when P-gp was introduced into these cells by transfection (either permanent or transient) the volume-activated chloride currents acquired TPA sensitivity. Second, the effects of PKC on channel activation were altered by the introduction of mutations into the putative phosphorylation sites in the linker region of P-gp. This latter result implies that the effects of PKC on channel activity not only require the presence of P-gp but are mediated through phosphorylation of P-gp. P-gp can be phosphorylated in the 'linker' region *in vivo* and *in vitro* (Chambers *et al.*, 1993; Orr *et al.*, 1993; Ahmad *et al.*, 1994). Although it has been reported that increased activation of PKC correlates with increased drug transport by P-gp, it has not yet been shown that this is due to phosphorylation of P-gp, or whether the phosphorylation sites in the linker region are involved. Thus, the present data provide the first evidence of a functional role for these phosphorylation sites and for the linker region of P-gp: phosphorylation of P-gp plays a role in regulating cell volume-activated channels.

It is important to note that PKC and P-gp are not required for channel activation by hypotonicity *per se*. Currents can be activated by hypotonicity in the presence of the PKC inhibitor peptide, in cells where no P-gp is detectable, as well as in cells in which the phosphorylation sites of P-gp are mutated. Instead, PKC and P-gp appear to regulate the magnitude of the currents elicited in response to hypotonicity. This is apparently in contrast to the role of phosphorylation in the activity of CFTR. CFTR is closely related to P-gp (Hyde *et al.*, 1990), and the R-domain of CFTR has been considered analogous to the linker region of P-gp: it is located in the same position between the two halves of the molecule and is the major site of phosphorylation. However, CFTR is itself a channel and phosphorylation of the R-domain is directly responsible for channel opening and closing (Cheng *et al.*, 1991; Rich *et al.*, 1991; Chang *et al.*, 1993). This is in contrast to phosphorylation of the linker region of P-gp which regulates the activation of an endogenous volume-activated chloride channel. The mechanisms by which this is achieved are unknown.

Channel or channel regulator?

In several cell types, increased expression of P-gp has been found to be associated with an increase in the magnitude of volume-activated chloride currents (Gill *et al.*, 1992; Valverde *et al.*, 1992; Altenberg *et al.*, 1994; Luckie *et al.*, 1994). This result can be interpreted in two alternative ways: either P-gp is itself the volume-activated chloride channel, or it is a regulator of an endogenous chloride channel. The experiments described here distinguish between these two hypotheses and provide strong support for the latter hypothesis. The finding that P-gp is a channel regulator does not exclude the possibility that it is also a channel, but the present data suggest that it

is not necessary to postulate that it possesses intrinsic channel activity.

HeLa cells exhibit substantial volume-activated chloride currents yet we were unable to detect P-gp in these cells. This suggests that a protein other than P-gp is the endogenous channel protein in HeLa cells. Instead, P-gp was shown to be a regulator of these endogenous currents: the endogenous currents in HeLa cells were insensitive to PKC activation but acquired sensitivity when P-gp was expressed in these cells. Additionally, the magnitude of the volume-activated currents did not increase when P-gp was expressed in HeLa cells, consistent with P-gp acting as a channel regulator, rather than being the channel protein itself.

The electrophysiological properties and pharmacology of the chloride currents associated with P-gp expression (for example in NIH-3T3*MDR1* cells) are indistinguishable from those of HeLa cells (Diaz *et al.*, 1993; this study). Given this similarity, it seems highly likely that the same channel protein is responsible for the chloride currents in both cell types. Untransfected NIH-3T3 cells exhibit small volume-activated chloride currents and expression of P-gp confers PKC sensitivity on these currents. In these cells P-gp expression also generates a significant increase in the magnitude of volume-activated chloride currents. Although this could be due to intrinsic channel activity of P-gp this now seems unlikely, as the characteristics of the P-gp channel would have to be indistinguishable from those of the endogenous volume-activated chloride channel in HeLa cells. More probably, P-gp enhances endogenous channel activity in NIH-3T3 cells in addition to imposing PKC sensitivity. If P-gp does enhance endogenous channel activity then these channels must be present in untransfected NIH-3T3 cells. That this is the case is shown by the observation that volume-activated chloride currents of greater magnitude than those elicited by standard osmotic gradients can be induced when greater osmotic gradients are used (Luckie *et al.*, 1994; M.A.Valverde, unpublished results). Thus, not only does P-gp expression confer PKC-sensitivity on volume-regulated chloride channels, it appears to enhance channel activity by reducing the osmotic gradient required to activate these channels. Whether these two regulatory effects are related, or distinct, is not yet known.

Until the molecular identity of the channel underlying the volume-activated currents studied here has been identified, the mechanism by which phosphorylation of the linker region of P-gp regulates these channels remains speculative. It is also not yet clear how the drug transport and channel regulator activities of P-gp are related. Although drug transport can influence channel activity (Gill *et al.*, 1992), and vice versa (Sardini *et al.*, 1994), it is clear that the two processes are distinct and separable (Gill *et al.*, 1992; Mintenig *et al.*, 1994). This is an area of current investigation. Nevertheless, the finding that phosphorylation of P-gp regulates volume-activated chloride channels provides new insights into the function of P-gp and its potential role in cell volume regulation.

Materials and methods

Cells

NIH-3T3*MDR1* cells are NIH-3T3 fibroblasts permanently transfected with human *MDR1* cDNA (Pastan *et al.*, 1988; Shen *et al.*, 1986); these cells stably overexpress P-glycoprotein and are multidrug resistant. HeLa cells were obtained from the ICRF cell culture facilities at Clare Hall, UK. Cells were cultured in 35 mm plastic dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For NIH-3T3*MDR1* cells the medium was also supplemented with 1 μg/ml colchicine. Cells were used within 48 h of subculturing.

Electrophysiological recordings

Whole-cell chloride currents were measured by the patch-clamp technique as described previously (Sheppard *et al.*, 1991; Gill *et al.*, 1992). The standard pipette solution contained (in mM): 140 *N*-methyl-D-glucamine chloride (NMDGCl), 1.2 $MgCl_2$, 1 EGTA, 2 ATP, 10 HEPES, pH 7.4; the isotonic bathing solution contained 140 NMDGCl, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, pH 7.4. Hypotonic bathing solution was identical to the isotonic solution except that the NMDGCl concentration was reduced to 105 mM. The tonicities were measured by freezing point depression and adjusted with mannitol to 280 mOsm for the pipette, 300 mOsm for the isotonic bathing solution and 220 mOsm for the hypotonic bathing solution. Error bars on the figures represent SEM. Where appropriate, unpaired Student's *t*-tests were used to assess the significance of differences between data points.

Mutagenesis and transient transfections

Oligonucleotide-directed mutagenesis of human *MDR1* cDNA was performed using the Altered Sites *in vitro* mutagenesis system (Promega). Plasmid pMDR7 expressed the human *MDR1* gene under control of the phage T7 promoter (Valverde *et al.*, 1992). For mutagenesis, a 2199 bp *Eco*RI–*Pst*I fragment from the *MDR1* cDNA (nucleotides 1601–3800) was cloned between the *Eco*RI–*Pst*I sites of pAlter-1 (Promega) to generate plasmid pSRP. Mutagenesis was performed using oligonucleotides 3′-CAAATGATTCAAGAGCCGCCCTAATAAGAAAAA-GAGCCGCCCGTAGGGCCGTCCGTGGAGCCCAAGCCCAAGACA-GAAAGCTTGCCGCCAAAGAGGCTCTGG-5′ to construct the P-gp8A mutant and 3′-CAAATGATTCAAGAGAGGAGCTAATAAGAA-AAAGAGAGGAGCGTAGGGAGGTCCGTGGAGAGCAAGCCCAA-GACAGAAAGCTTGAGGAGAAAGAGGCTCTGG-5′ to construct the P-gp8E mutant. The entire 2199 bp fragment of pSRP was sequenced after mutagenesis to ensure that only the intended changes had been introduced. The mutated fragments from pSRP were then recloned between the *Eco*RI and *Pst*I sites of pMDR7 to generate plasmids pMDR737 (P-gp8A) and pMDR738 (P-gp8E), respectively.

Transient expression of wild type or mutated P-gp in NIH-3T3 or HeLa cells was achieved using the vaccinia-based, T7-polymerase system as described previously (Gill *et al.*, 1992). Transfected cells for electrophysiological studies were selected from the population based on changes in cell shape which are a consequence of vaccinia infection.

Chemicals

Phorbol 12-myristate 13-acetate (TPA) was obtained from Sigma and stored as stock solutions in dimethyl sulfoxide at −70°C. The PKC pseudosubstrate inhibitory peptide and control peptide were kindly donated by Dr M.Crumpton (ICRF) and stored in water at −20°C.

Western blotting

Proteins were extracted by washing cell suspensions twice in PBS and solubilizing the cell pellet with an equal volume of 1% (v/v) Triton X-100 in 10 mM Tris–HCl, pH 8.0 for 20 min on ice. Insoluble cell debris was removed by centrifugation and proteins solubilized at 37°C in Laemmli sample buffer (Laemmli, 1970). Proteins were quantified using the BCA protein assay (Pierce, IL, USA), separated by electrophoresis on a 6% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond, Amersham; Towbin *et al.*, 1979). P-glycoprotein was identified by probing with the monoclonal antibody C219 (Centercor Europe, Tongeren, Belguim) and chemiluminescence (ECL; Amersham, UK).

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