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P-glycoprotein and Cell Volume-Activated Chloride Channels

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The multidrug resistance P-glycoprotein (P-gp) is an active drug transporter which can expel hydrophobic compounds from cells. Expression of P-gp has many effects on cells and tissues and the physiological function, or functions, of P-gp are still unclear. Recently, expression of P-gp has been associated with altered activity of chloride channels which play a role in regulating cell volume of response to osmotic shock or nutrient uptake. The nature and physiological role of this association has been a subject of some debate. In this article, mechanisms by which P-gp might influence cell volume-activated chloride currents is discussed, and the potential physiological role of this regulation considered.

KEY WORDS: P-glycoprotein; chloride channels; cell volume regulation.

INTRODUCTION

The multidrug resistance P-glycoprotein is an active transporter which utilizes the energy of ATP hydrolysis to pump hydrophobic compounds out of cells, reducing their intracellular concentration (Gottesman and Pastan, 1993). Substrates that can be transported by P-gp include many cytotoxic drugs and, hence, expression of P-gp can confer resistance of cancers to chemotherapy. P-gp is a typical member of the ABC (ATP-binding cassette) family of transporters and consists of four domains, two hydrophobic domains which span the membrane multiple times, and two ATP-binding domains which couple ATP hydrolysis to the transport process (Fig. 1). (Hyde *et al.*, 1990). Between the two halves of the molecule is a short, charged region, the "linker" region, which is the major site of phosphorylation of P-gp.

P-glycoprotein can transport a wide variety of hydrophobic compounds and is expressed in many tissues and cell types. Consequently, many physiological functions for P-gp have been proposed, including cell detoxification, the removal of nonlipid compounds from membranes, hormone secretion, and control of phospholipid distribution. Furthermore, cells overexpressing P-gp show many biochemical changes, although it is not always clear which of these are primary and which are secondary consequences of P-gp expression. Thus, the physiological role(s) of P-gp are still unclear. However, an understanding of the physiological role(s) of this protein is important for the development of treatments which reverse multidrug resistance: compounds which inhibit the drug transport function of P-gp might exhibit side-effects of a consequence of inhibiting its normal physiological function(s). The recent and unexpected finding that expression of P-gp can influence cell volume-activated chloride currents (Valverde *et al.*, 1992) has, potentially, important implications for its physiological role.

P-GLYCOPROTEIN IS ASSOCIATED WITH CELL VOLUME-ACTIVATED CHLORIDE CURRENTS

In many cell types, characteristic chloride currents can be activated by changes in cell volume induced by changes in the osmolarity of the medium (Hoffmann

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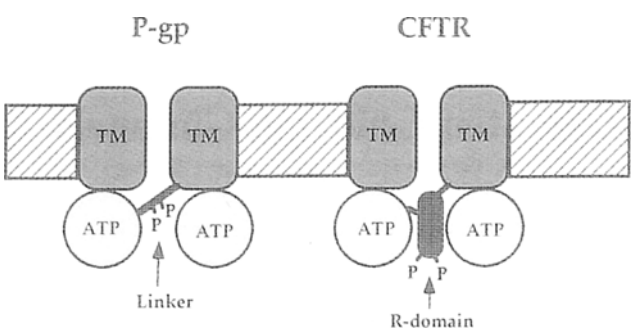

Fig. 1. Domain organization of P-gp and CFTR. Each protein, in common with other ABC transporters, has two transmembrane domains (TM) which span the membrane multiple times and two ATP-binding domains (ATP). Between the two halves of each protein are the major regions of phosphorylation, the "linker" region of P-gp and the "R-domain" of CFTR. CFTR is a channel, and phosphorylation of the linker region regulates channel opening and closing. In contrast, P-gp appears to be a channel regulator, and phosphorylation of the linker region influences the activity of a distinct channel protein. Thus, although the "linker region" and "R-domain" are superficially similar, they may serve different functions in the two proteins.

and Simonsen, 1989). A line between P-gp expression and these cell volume-activated chloride currents was first identified in NIH3T3 fibroblasts (Valverde *et al.*, 1992). In NIH3T3 cells, the magnitude of cell volume-activated chloride currents is relatively small, but is considerably larger in cells which have been permanently transfected with human *MDR1* cDNA expressing P-gp (Valverde *et al.*, 1992). Similar results have been reported subsequently (Gill *et al.*, 1992; Altenberg *et al.*, 1994; Luckie *et al.*, 1994; Zhang *et al.*, 1994; Hardy *et al.*, 1995), although in other studies an association between P-gp expression and cell volume-activated chloride currents was not observed (Rasola *et al.*, 1994; Wang *et al.*, 1994; Dong *et al.*, 1994; Hardy *et al.*, 1995). One caveat which must be applied to all these studies is that the phenotype of permanently transfected cell lines may diverge from that of the parent cell line after many passages in cell culture. Thus, any difference (or lack of difference) between a permanently transfected line and its parent may be due to a difference other than P-gp. A clear association between P-gp expression and the magnitude of cell volume-activated chloride currents was obtained by transient expression of P-gp, and by ablation of P-gp expression using antisense oligonucleotides (Valverde *et al.*, 1992). Several other lines of evidence are also consistent with an association between P-gp and channel activity. Although individually none of these studies prove a link between P-gp and channel activity, taken together the evidence is compelling. First, compounds such as verapamil, dideoxyforskolin, nifedipine and quinine, which interact with P-gp and inhibit drug transport, also block cell volume-activated chloride currents (Valverde *et al.*, 1992; Mintenig *et al.*, 1993). Second, cytotoxic drugs which can be transported by P-gp, such as daunomycin and vincristine, prevent channel activation. Inhibition is only observed when the drug can be transported (i.e., is added to the intracellular face of the membrane in the presence of hydrolyzable ATP), suggesting that drug transport by P-gp prevents channel activation (Gill *et al.*, 1992). Third, monoclonal antibodies against P-gp block cell volume-activated chloride currents (Zhang and Jacob, 1994; Han *et al.*, 1995). Fourth, activation of chloride channels by cell swelling inhibits P-gp-dependent drug transport (Sardini *et al.*, 1994). Finally, expression of P-gp and the cystic fibrosis gene product (CFTR) appears to be coordinately regulated: CFTR is known to be a chloride channel (Trezise *et al.*, 1992; Breuer *et al.*, 1994). Thus, there can be little doubt that P-gp expression is associated with cell volume-activated chloride channels: the question is, how does P-gp expression influence channel activity?

CHANNEL OR CHANNEL REGULATOR?

The finding that P-gp expression can increase the magnitude of cell volume-activated chloride currents can, potentially, be interpreted in two ways. The simplest hypothesis is that P-gp has intrinsic channel activity (i.e., is the channel protein itself) and that expression of P-gp simply generates more channel proteins and hence an increase in the magnitude of the currents. Initially, this hypothesis seemed most plausible, not only because it was the simplest hypothesis consistent with the available data, but also because the closely related cystic fibrosis gene product, CFTR (Fig. 1), has intrinsic channel activity (Anderson *et al.*, 1991; Bear *et al.*, 1992). However, the possibility that P-gp is a channel regulator, lacking intrinsic channel activity but regulating an endogenous volume-activated chloride channel, is also consistent with these data. In the past two years, considerably effort has been invested in trying to distinguish between these alternatives. The available data now strongly favor the latter hypothesis and

suggest that P-gp is a channel regulator, rather than being the channel protein itself.

THE MOVEMENT OF CHLORIDE IONS IS NOT COUPLED TO DRUG TRANSPORT

P-gp can transport hydrophobic compounds, and a potentially trivial explanation of the observation that P-gp is associated with chloride ion movement is that chloride ions simply move across the membrane together with transported substrates. However, several lines of evidence show that drug transport and chloride ion movement are distinct and separable processes. Drug transport requires ATP hydrolysis by P-gp, while nonhydrolyzable ATP analogues can substitute for ATP in channel activation (Gill *et al.*, 1992). Mutations can be introduced into P-gp which prevent drug transport but have little effect on channel activity (Gill *et al.*, 1992). Compounds have been identified which block either drug transport or channel activity, but not both (Mintenig *et al.*, 1993). Finally, coupled transport of drugs and chloride ions is inconsistent with the demonstration of drug transport in the absence of chloride (Altenberg *et al.*, 1994a), and the finding that channel activation inhibits drug transport from cells (Sardini *et al.*, 1994).

P-GLYCOPROTEIN MAY EXIST IN DISTINCT CONFIGURATIONS ASSOCIATED WITH DRUG TRANSPORT AND CHLORIDE CHANNEL ACTIVITY

Two lines of evidence suggest that the drug transport and chloride channel activities associated with P-gp may be mutually exclusive, at least under some circumstances. First, cytotoxic drugs which are transported by P-gp, such as daunomycin and vincristine, inhibit the activation of P-gp-associated chloride currents (Gill *et al.*, 1992; Nilius *et al.*, 1994). These drugs are not channel blockers, but prevent activation of the channel by cell swelling (Mintenig *et al.*, 1993). Importantly, these drugs only inhibit channel activation when they can be transported: when they are added to the intracellular face of the membrane (the side of the membrane from which they are normally transported), when hydrolyzable ATP is present, but not when P-gp is mutated so it can no longer hydrolyze ATP (Gill *et al.*, 1992). Second, channel activation by cell swelling in response to hypotonicity inhibits P-gp-dependent drug transport and, when cells are returned to isotonicity and cell volume restored, drug transport is also restored (Sardini *et al.*, 1994). This apparent exclusivity of the channel and transporter activities associated with P-gp may reflect two distinct configurations of the protein. Whether these are conformational configurations, reflect different associations with other cellular components, or are a consequence of protein modification (e.g., phosphorylation) is not known.

P-gp IS A CHANNEL REGULATOR

In several studies, expression of P-gp has been found to increase the magnitude of cell volume-activated chloride currents. This could be due to an intrinsic channel activity of P-gp itself, or to increased activation of latent, endogenous channels. However, a correlation between P-gp levels and chloride currents is by no means always observed, and in several cell types which do not express P-gp at detectable levels, large volume-activated chloride currents can be activated (Rasola *et al.*, 1994; Wang *et al.*, 1994; Dong *et al.*, 1994; Hardy *et al.*, 1995). Although it is often inappropriate to compare data obtained with different cell types and different experimental protocols, it is not easy to accommodate these latter data within a model in which P-gp is itself the channel. Instead, such data imply that any association between P-gp and chloride channel activity must be regulatory. Recent data provide direct evidence for such a regulatory association (Hardy *et al.*, 1995).

P-gp contains a number of consensus protein kinase C (PKC) phosphorylation sites, located in the "linker" region between the two halves of the molecule in an analogous position to the phosphorylated R-domain of CFTR (Fig. 1). Several of these sites can be phosphorylated by PKC, both *in vivo* and *in vitro* (Chambers *et al.*, 1993; Orr *et al.*, 1993). Activation of PKC in P-gp expressing cells, for example by treatment with phorbol esters, prevents activation of cell volume-activated chloride currents. However, in cells which do not express P-gp, yet exhibit typical volume-regulated chloride currents (e.g., Hela cells; Diaz *et al.*, 1993), these currents are insensitive to PKC activation. Expression of P-gp confers PKC-sensitivity on channel activation in these cells. This appears to be a consequence of phosphorylation of the "linker"

region of P-gp as mutation of these phosphorylation sites abolishes any effect. Thus, PKC regulation of chloride channels can be mediated through phosphorylation of P-gp, showing that P-gp is a regulator of endogenous channel proteins. Whether this regulation will be apparent in all cell types, or whether some cells lack the appropriate PKC isoforms or signalling pathways, remains to be determined. Although the conclusion that P-gp is a channel regulator does not exclude the possibility that P-gp also possesses intrinsic channel activity (either for chloride or for another ion), it is not necessary to invoke intrinsic channel activity in order to account for the observed influence of P-gp on channel activity.

It has been suggested that phosphorylation of the "linker" region of P-gp might regulate its transport activity, although such an effect has yet to be demonstrated. Indeed, mutation of P-gp so that the "linker" region can no longer be phorphorylated does not appear to influence drug transport significantly (H. Goodfellow and CFH, unpublished results). If phosphorylation does not influence drug transport, then these conserved phosphorylation sites presumably serve an alternative physiological function. This is, potentially, provided by the finding that phosphorylation of the "linker" region influences the activation of volume-regulated channels.

The observation that phosphorylation of the "linker" region of P-gp can influence channel activity is reminiscent of the role of the R-domain of CFTR.

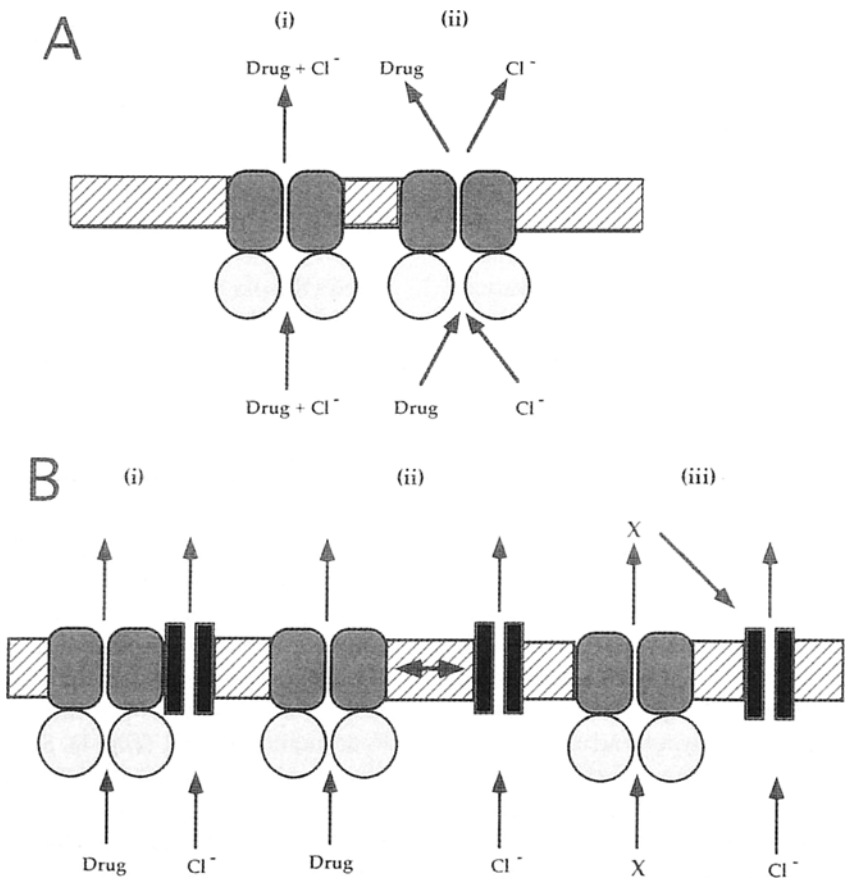

Fig. 2. Possible mechanisms by which P-gp might influence channel activity. (**A**) P-gp has intrinsic channel activity. Two extremes of this hypothesis can be envisaged: (i) the channel and transport activities are coupled (ions move across the membrane with drugs and vice versa); (ii) the movements of drugs and ions are entirely independent. (B) P-gp is a channel regulator. (i) P-gp could interact directly with the channel protein (black) to modulate its activity; (ii) P-gp could influence the channel indirectly through an intermediate cellular component involved in volume regulation such as the cytoskeleton or membrane; (iii) P-gp could transport a regulatory molecule (X) which influences channel activity.

The R-domain is located in an analogous position within CFTR to the "linker" region of P-gp (Fig. 1); it is the major site of CFTR phosphorylation and regulates CFTR channel activity (Cheng *et al.*, 1991; Rich *et al.*, 1991; Chang *et al.*, 1993). However, CFTR is itself a channel, and phosphorylation of the R-domain mediates channel opening and closing in an intramolecular reaction. In contrast, phosphorylation of the "linker" region of P-gp is involved in intermolecular regulation, influencing channel activity mediated by a distinct cellular protein. Thus, although the "linker region" and "R-domain" are superficially similar, they may serve distinct functions in the two proteins.

HOW DOES P-gp REGULATE CHANNEL ACTIVITY?

P-gp can exert two distinct effects on cell volume-activated chloride currents. First, in some cell types, P-gp expression increases the magnitude of these currents. This effect appears to be due to an alteration in the osmotic gradient required for channel activation (Luckie *et al.*, S. Hardy, M. A. Valverde, and C. F. Higgins, unpublished data). Thus, in P-gp expressing cells, a smaller osmotic gradient is required to activate currents of a given magnitude than in non-P-gp-expressing cells. Second, P-gp expression can confer PKC regulation on channel activation. Whether these two effects are independent consequences of P-gp expression, or are manifestations of the same regulatory process, has yet to be determined.

Several possible mechanisms by which P-gp might regulate channel activity can be envisaged (Fig. 2), although many more data are required before it will be possible to distinguish between them. The simple hypothesis, that P-gp transports a regulatory molecule (X) which modulates channel activity (Fig. 2B,iii), can be excluded: when P-gp is mutated such that its transport function is severely impaired it still regulates channel activity (Gill *et al.*, 1992), and compounds which inhibit the transport function of P-gp do not necessarily impair its ability to influence channel activity (Mintenig *et al.*, 1993). Alternative mechanisms by which P-gp might influence channel activity include a direct interaction between P-gp and the channel protein (Fig. 2B,i), indirect effects on insertion of the channel protein into the membrane, or cell volume-sensing mechanisms involving the cytoskeleton or membrane lipids. (Fig. 2B,ii). A resolution of this point will probably require identification of the channel which is regulated by P-gp (see below).

PHARMACOLOGY OF P-gp AND VOLUME-ACTIVATED CHLORIDE CURRENTS

Many chemically diverse compounds which interact with P-gp and inhibit P-gp-dependent drug transport also inhibit cell volume-activated chloride currents. This, of course, has implications for the reversal of multidrug resistance in the clinic and its potential side-effects. These compounds can be divided into four classes (Mintenig *et al.*, 1993). Class I compounds are substrates which are transported by P-glycoprotein. These compounds do not block the channel but prevent channel activation. This appears to be a consequence of their transport, as they only prevent channel activation when added to the intracellular face of the membrane and when hydrolyzable ATP is present (see above).

Class III compounds (such as DIDS and NPPB) block cell volume-activated chloride currents but do not inhibit P-gp-dependent drug transport, while Class IV compounds (the cyclosporins) block drug transport but do not affect channel activity. Thus, the drug transport and channel functions associated with P-gp can be separated, pharmacologically.

Class II compounds inhibit drug transport by P-gp and also inhibit volume-activated chloride currents. They act independently of the transport function of P-gp and block from the extracellular surface of the membrane. The most potent and specific of these is tamoxifen (Valverde *et al.*, 1993; Zhang *et al.*, 1994). Although the effects of Class II compounds on channel activity are consistent with those of true channel blockers, it is not yet known whether their target is the channel itself, or another cellular component. Intriguingly, most compounds which bind to P-gp and reverse P-gp-mediated multidrug resistance, including verapamil, dideoxyforskolin, and tamoxifen, also block the P-gp-associated chloride channel. The simple interpretation of this finding, that Class II compounds influence channel activity as a consequence of their interaction with P-gp, may not be generally applicable. At least some Class II compounds, including tamoxifen, can inhibit channel activity in the absence of P-gp and must therefore interact with an alternative target. As Class II compounds are chemically diverse, it is interesting to

consider how a similar spectrum of compounds can inhibit both P-gp-mediated drug transport and channel activity, yet show little activity against other chloride channels. Do these compounds influence the transport activity of P-gp and channel activation though a single common target? Perhaps an appropriate interaction with lipids is a necessary prerequisite to their effects on P-gp and the channel protein. Do P-gp and the channel protein have structurally similar sites at which these compounds can interact? Or, is the similar pharmacology of P-gp and channel activity simply a coincidence?

OTHER ABC TRANSPORTERS MAY BE CHANNEL REGULATORS

P-gp can regulate volume-activated channels, at least under certain circumstances. P-gp is an ABC transporter and there is increasing evidence that other ABC transporters may also regulate ion channel function. The MRP protein, an ABC transporter which can also confer multidrug resistance, influences both chloride and potassium currents in cells in which it is expressed (Jirsch *et al.*, 1993, 1994). The CFTR protein, in addition to its intrinsic ion channel activity, is also reported to modulate the activity of another chloride channel (the outwardly rectifying chloride channel, ORCC; Egan *et al.*, 1993; Gabriel *et al.*, 1993). The ATP-sensitive potassium channel of pancreatic β-cells is sensitive to sulfonyl ureas, yet the sulfonyl urea receptor may be distinct from the channel, an ABC transporter which regulates the channel protein (Ashford *et al.*, 1994; Bryan, 1994). Finally, the Trk potassium channel of *E. coli* is regulated by an ABC transporter; this regulation appears to be independent of its transport activity, a situation highly analogous to that of P-glycoprotein and the volume-activated chloride currents (Parra-Lopez *et al.*, 1994).

CELL VOLUME REGULATION: A PHYSIOLOGICAL ROLE OF P-gp?

The cell volume-activated chloride currents associated with P-gp are similar in anion selectivity, voltage sensitivity, time-dependent decay, and pharmacology to those currents believed to provide the rate-determining step in cell volume regulation in many cell types. This raises the question of whether P-gp has a physiological role in cell volume regulation.

Most, if not all cells can regulate their volume (Hoffman and Simonsen, 1989; Sarkadi and Parker, 1991). This is frequently achieved by activating ion channels in the cell membrane in response to osmotic imbalance: the movement of ions is followed by a corresponding movement of water which restores cell volume. Although cell volume-regulated chloride channels have been characterized extensively, using electrophysiological techniques, the molecular identity of the channel protein(s) is obscure. A putative chloride channel (pI_{Cln}) was cloned by expression in *Xenopus* oocytes (Paulmichl *et al.*, 1992). Although, like P-gp, expression of pI_{Cln} increased chloride currents, subsequent studies showed that pI_{Cln} is located in the cytosol and is probably a channel regulator rather than the channel itself (Krapivinsky *et al.*, 1994). The only other protein linked to volume-regulated chloride currents is ClC-2 (Thiemann *et al.*, 1992; Grunder *et al.*, 1992). Although there is little doubt that ClC-2 has intrinsic chloride channel activity, the channel differs in voltage sensitivity, anion selectivity, and pharmacological properties from the swelling-induced chloride conductances which are associated with P-gp and mediate cell volume regulation. Thus, the channel protein behind the volume-regulated currents remains to be identified.

P-gp is not essential for cell volume regulation as cells which lack P-gp can still perform this function. However, as P-gp can regulate the activity of channels which play an important role in this process, P-gp may modulate cell volume regulation. One of the difficulties in studying cell volume regulation is mimicking physiological conditions: that the magnitude of osmotic gradient required for *in vitro* studies is much greater than that normally experienced by cells *in vivo*. The development of more sensitive methods for measuring the rate and magnitude of cell volume changes may be necessary before the role of P-gp can be ascertained. It may be significant, however, that expression of the genes encoding P-gp and CFTR appears to be coordinately regulated (Trezise *et al.*, 1992; Breuer *et al.*, 1994): CFTR is involved in the regulation of fluid movements across epithelia.

PROSPECTS AND CONCLUSIONS

Two major factors limit our understanding of how P-gp might regulate volume-activated currents. First, the identity of the channel protein is still obscure. Indeed, it is not even known whether a

single protein species is responsible for these volume-regulated currents in different cells, or whether there is a multigene family, and related channel proteins with slightly different characteristics are expressed in different cell types. Second, the P-gp-associated currents have not been characterized at the single-channel level. Although single-channel recordings related to volume-activated currents have been reported (Solc and Wine, 1991), it has not been possible to demonstrate, unambiguously, that these or any other channels underlie the P-gp-associated cell volume-activated responses. This is primarily because of difficulties in reproducibility activating such channels in isolated membrane patches, mimicking the effects of cell swelling.

Although a physiological role for P-gp in cell volume regulation has not been established, the relationship between P-gp and chloride channel activity may have general ramifications, not only for the physiological role of P-gp, but for cell volume regulation, channel regulation, and the reversal of multidrug resistance in cancer chemotherapy. If the relationship between P-gp and channel activity is to be elucidated, an important challenge is to identify the channel protein and its gene. The association with P-gp itself may provide a means whereby the channel can be identified. Future developments are awaited with interest.

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