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DRUGS TRANSPORTED BY P-GLYCOPROTEIN INHIBIT A 40pS OUTWARDLY RECTIFYING CHLORIDE CHANNEL

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SUMMARY: P-glycoprotein functions as an ATP-dependent pump for a diverse spectrum of compounds. Recently, it has been shown that P-glycoprotein may be bi-functional and act as a chloride channel as well as a pump. The single channel properties of this conductance are unknown, however, as macroscopic, whole cell currents are inhibited by substrates for P-glycoprotein transport, the single channels underlying this response should also be blocked by these compounds. We found that colchicine, vinblastine, daunomycin and verapamil (50μM) caused block of a 40 pS outwardly-rectifying chloride channel in cells expressing P-glycoprotein. The inhibitory effect of these compounds appeared specific for the 40 pS chloride channel as a large, 300 pS chloride channel found in the same cells was unaffected by addition of drug. These results suggest that the 40 pS chloride channel may be associated with P-glycoprotein expression.

P-glycoprotein, the protein product of the multidrug resistance gene (mdr1) acts as an ATP-dependent pump which extrudes various substrates, including chemotherapeutic drugs from the cytosol of cells [1]. Although the natural substrates for P-glycoprotein have not been identified, it has been proposed that P-gp normally acts to eliminate cellular toxins [2]. The research groups of Sepulveda and Higgins [3,4] recently provided evidence which suggests that P-gp may be bifunctional and possesses channel activity in addition to transporter function. They found that transfection of mdr1 into fibroblasts which do not endogenously express the gene led not only to drug transport activity but also the appearance of a swelling-activated Cl^- conductance path. This conductance is dependent on ATP binding and is inhibited by substrates for P-glycoprotein transport activity. So far, only the macroscopic properties of the Cl^- conductance conferred with mdr1 transfection have been examined [3,4]. A detailed description of the P-glycoprotein-associated channel based on single channel data is not available. Hence, it is not yet possible to prove identity between P-glycoprotein and a particular Cl^- channel. However, we would expect on the basis of the available macroscopic data, that the P-gp associated channel would possess an outwardly-rectifying current-voltage relationship, be stimulated by cell swelling, modulated by ATP binding and inhibited by substrates of transporter function in an ATP-dependent manner [3,4].

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A chloride selective, 40 pS, outwardly rectifying channel has been observed in several different types of epithelial cell and also in some nonepithelial cells [5-7]. The means of activation of this channel remains somewhat controversial. The channel cannot be consistently activated in patch clamp studies of the intact cell. However in some cases, this channel has been stimulated by cell swelling. Solc et.al. observed both the 40 pS and a larger, 80 pS, outwardly rectifying chloride channel stimulated by swelling of airway epithelial cells [8]. Solc speculated that the smaller conductance channel may represent a subconductance level of the 80 pS channel. In excised, inside-out membrane patches, it is activated consistently with the application of large, nonphysiological, depolarizing voltage steps of at least 80 mV [5]. It has been reported by two research groups that this outwardly-rectifying Cl^- channel can also be activated by PKA-phosphorylation in cell membranes which co-express CFTR [9,10], the protein product of the Cystic Fibrosis gene.

As the 40 pS channel has been implicated as a candidate for swelling-activated chloride currents, we sought to examine its relationship to P-glycoprotein. According to Gill et.al. [4] and Diaz et.al.[11], swelling-activated, whole-cell chloride currents, associated with P-glycoprotein expression are inhibited in an ATP-dependent manner by substrates for transport by P-glycoprotein. The purpose of this study was to investigate the effect of substrates for transport by P-glycoprotein on activity of the 40 pS, outwardly-rectifying chloride channel.

MATERIALS AND METHODS

Cell Culture of CHO Cells: The culture conditions for the parental chinese hamster ovary (CHO) cells (Aux B1) and the multidrug resistant CHO cell line selected for colchicine resistance (B30), have been described previously [12].

Single Channel Studies: Single channel currents in excised, inside-out membrane patches were measured according to Hamill et.al.[13] using a List EPC-7 patch-clamp amplifier (Medical Systems, Great Neck, N.Y.) Pipettes were fabricated from borosillicate glass type 7052 (Garner Glass Co.) using a two-stage Narishige pipette puller. When filled with Na^+-hepes solution, pipette resistances were approximately 2-5 MΩ. Current output was monitored on a Tektronix oscilloscope and stored on video tape after A/D conversion by a video adaptor (PCM 2, Medical Systems). Recorded single channel currents were transferred to the hard disk of an IBM-AT compatible computer and played back for analysis using pCLAMP (V.V.1) software (Axon Instruments). During playback, single channel records were filtered using a 6-pole Bessel filter set at 500 Hz low pass frequency. Unitary current amplitudes of recorded data were measured by forming histograms of open-level data points and fitting those histograms with Gaussian curves using a least-squares algorithm. Event duration histograms were constructed from continuous records of single channel activity, lasting 60-180 s. using a 50% threshold criterion. *Solutions*: The standard bath and pipette solutions contained (mM): 140 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). The pH was adjusted to 7.2 using NaOH. Experiments were performed at 22-25'C.

RESULTS

Outwardly-rectifying chloride channels, with a unitary conductance of 40 pS around 0 mV (±20 mV) are found after activation by depolarization in 41 of 102 excised membrane patches obtained from CHO cells expressing high levels of P-glycoprotein (B30 cells) (12) and 3 of 25 patches obtained from the parental CHO cell line Aux B1. The difference in density of this

channel between the two cell lines suggests, but does not prove, that it is related to P-glycoprotein expression. As previously mentioned, the only manner in which to consistently activate this channel requires membrane patch excision with the application of large depolarizing voltages. We found that the 40 pS channel exhibited all of the properties previously reported in studies of epithelial cells [5]. Namely, the current-voltage relationship of the channel was outwardly-rectifying in symmetrical chloride solutions and it showed voltage dependence with increased probability of opening at depolarized potentials.

In order to determine whether the 40 pS outwardly-rectifying chloride channel is related to P-glycoprotein expression, we sought to assess the inhibitory effect of known substrates for P-glycoprotein transport. According to the hypothesis proposed by Gill et.al., if the 40 pS channel underlies the whole-cell currents previously shown to be associated with P-glycoprotein expression, these substrates should inhibit its function in a manner dependent on ATP hydrolysis. We chose to study the effect of colchicine, daunomycin, vinblastine and verapamil on channel activity as the efficacy of these drugs as substrates for P-glycoprotein

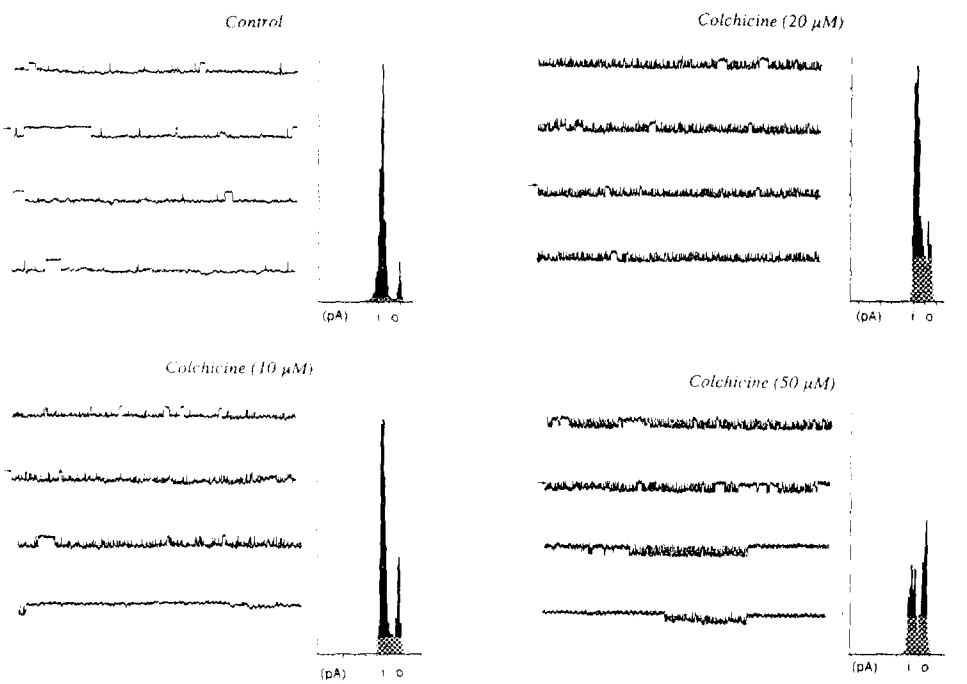

Figure 1. Dose Dependent Effect of Colchicine on Activity of 40 pS Chloride Channel. We examined the effect of varying concentrations of colchicine (0-50 μM) on activity of the 40 pS channel. In these experiments, colchicine was added to cell monolayers at the concentrations noted and following a 15 minute incubation time, the channel was studied in excised membrane patches after its activation by depolarization steps of 40-80 mV. Arrows show the current level of the closed state and downward deflections are indicative of inward chloride current at a holding potential of +40 mV. The upper left hand panel shows control channel activity. Colchicine (10-50 μM) treatment clearly induced blockade. Three to ten studies were performed at each concentration. Amplitude histograms are shown on the right and show the mean current levels corresponding to the closed (0) and open (i) states of the channel. The channel amplitude was not markedly different at 50 μM colchicine than in the control experiment. Low pass filter was 500 hz.

transport and as activators of P-glycoprotein ATPase activity has been well studied in B30 cells (14).

We first sought to determine whether pretreatment with colchicine (for a minimum of 15 minutes) affected our ability to activate the channel by membrane depolarization. We were unable to detect any difference between the times required for depolarization-evoked activation in untreated and colchicine-treated membranes (2-5 minutes in each case). However, once opened by depolarization we found that those channels pretreated with colchicine (10 to 50 μM) exhibited altered activity compared with untreated channels (figure 1). There were marked changes in intraburst channel kinetics. In untreated cells, open time histograms for the channel were fit best by a single exponential. In channels pre-exposed to colchicine, mean open time decreased from control values of 80± 70 (SD) ms to 14 ±14 (SD) ms and the open time histograms for the drug-treated channels were best fit using two exponentials (figure 2). On the

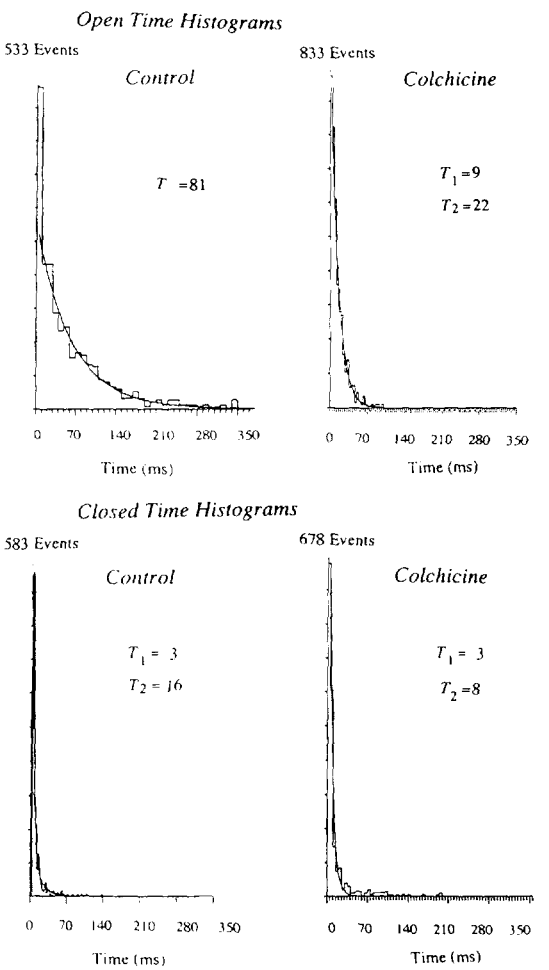

Figure 2. Treatment with Colchicine Alters Channel Kinetics. Open time and closed time histograms have been shown for 40 pS channel activity in control (untreated) and colchicine (50μM) treated B30 membranes. Introduction of a novel short-lasting open time constant probably accounts for the "flickery"-type blockade observed with colchicine-treated channels.

other hand, closed time histograms were not markedly affected by colchicine pretreatment. Therefore, colchicine acts to inhibit this channel primarily through the introduction of a novel, short lasting open time constant.

The results of the previous experiments do not allow us to determine at which side of the membrane the drug acts. Hence, we studied the effect of colchicine when added directly to the cytosolic surface of excised, inside-out membrane patches. We found that direct application (10 μM) induced a flickery block, similar to that evoked in the previous studies with intact cells (figure 3). The induction of channel block by colchicine in inside-outside membrane patches in ATP-free bath media suggests that ATP may not be required for channel block. In further support of this prediction, we found that addition of ATP (1mM) to the cytosolic face of colchicine-treated, inside-out membrane patches, caused no further change in channel kinetics (data not shown). Addition of colchicine (10 μM) to the outer surface of excised membrane patches, via the patch pipette, also caused a flickery-type channel block. Analysis of open duration histograms revealed that colchicine application to the outer membrane surface caused the appearance of a novel short-lived open state, T= 15 ms. These findings suggest that colchicine is capable of inhibiting this channel either from the extracellular or intracellular membrane surface.

We examined the effect of three other substrates for P-glycoprotein transporter activity on 40 pS channel activity, namely, daunomycin, vinblastine and verapamil. All three of these drugs caused channel block (figure 4) with an decrease in mean open time from control (80 ms) to 9±7 (SD) ms for daunomycin, 14 ±13 (SD) ms for vinblastine and 16±16 (SD) for verapamil. Analysis of open time histograms shows that block probably occurred through the generation of a second, short duration open state for all three substrates (figure 5).

In order to assess the specificity of the blockade caused by P-transport substrates on the 40 pS channel, we examined the kinetics of another chloride channel which appeared frequently in

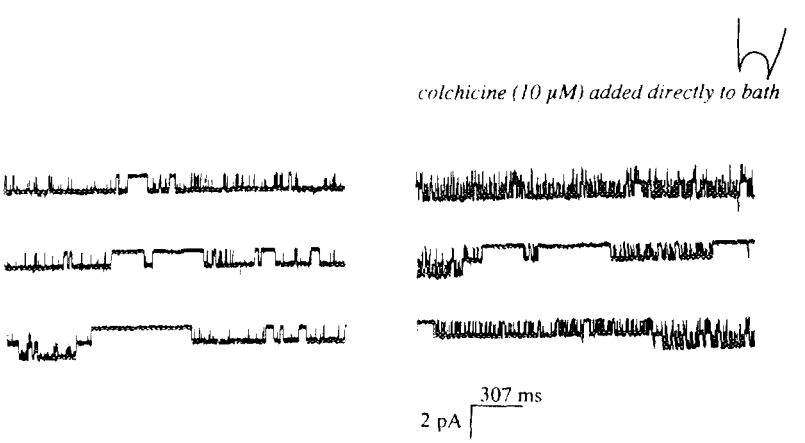

Figure 3. Inhibitory Effect of Colchicine In Inside-out Membrane Patches. Acute exposure of colchicine (10 μM) to the cytosolic surface of an inside-out patch induced channel block. This record is representative of four studies. The membrane patch was held at +40 mV.

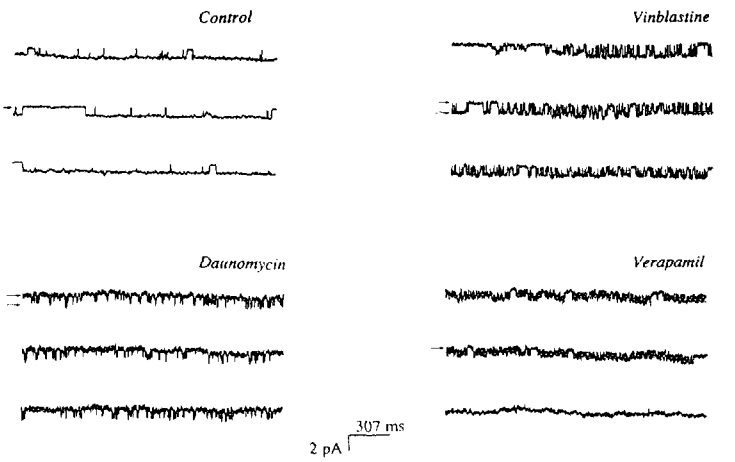

Figure 4. Inhibitory Effect of Vinblastine, Daunomycin and Verapamil on 40 pS Channel. Pretreatment of B30 cells with vinblastine (50μM), daunomycin (50 μM) and verapamil (50μM) caused block of 40 pS channel. These current records are representative of 5,4, and 6 studies,respectively. The arrow indicates the current level of the channel closed state.

B30 membranes. A 300 pS chloride channel is present in most excised patches and exhibits voltage dependence in that it is mostly open at 0 mV and open probability decreases as the potential is clamped at potentials higher than 20 mV, in either the depolarizing or hyperpolarizing direction. In contrast to our findings with the 40 pS channel, pretreatment with the drugs discussed previously did not have any effect on the kinetics of the 300 pS channel. Single channel data is shown for daunomycin-treated membranes in figure 6.

To further study the relationship of the 40 pS channel to P-glycoprotein, we studied the effect of an inhibitor of P-glycoprotein ATPase activity. We found that the addition of sodium orthovanadate (1 mM) to the cytosolic surface of inside-out patches did not cause any change in channel kinetics (data not shown).

DISCUSSION

On the basis of studies of whole cell currents by Diaz (11) and Gill (4) in mdr1-transfected cells, one would expect that the single channel currents associated with P-glycoprotein expression are inhibited by its transport substrates. The results shown in this paper show that four such substrates inhibit an outwardly-rectifying, 40 pS channel. All four compounds, colchicine, vinblastine, daunomycin and verapamil appear to block this channel by increasing the frequency of brief, nonconducting periods in the current traces. Together with the reports that this channel is active in the membrane of swollen epithelial cells (8) and its activity can be modulated by ATP (15), our observation provides further support for the argument that activity of this channel is associated with P-glycoprotein expression.

The mechanism for blockade of the 40 pS channel by substrates for P-glycoprotein transport is unknown. Gill et. al. (4) hypothesized that these drugs cause channel inhibition because they

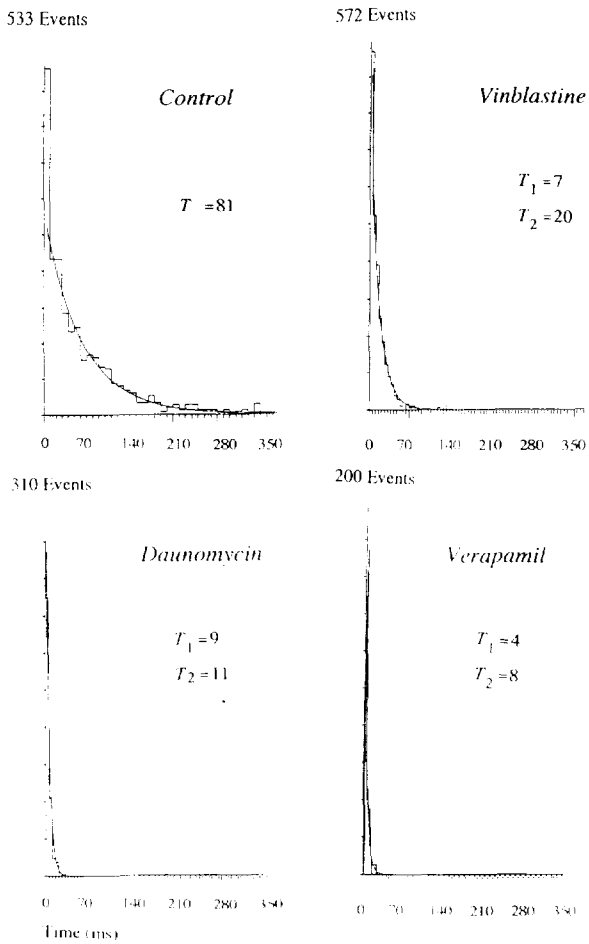

Figure 5. Kinetics of Block Caused by Drugs. Analysis of open time histograms reveal that all drugs examined, vinblastine, daunomycin and verapamil cause introduction of novel short-lasting open time constant.

stimulate P-glycoprotein ATPase activity thereby inducing P-glycoprotein to assume a "transporter" conformation rather than a "channel" conformation. In support of this hypothesis, we observed that sodium orthovanadate, an inhibitor of P-glycoprotein ATPase activity, did

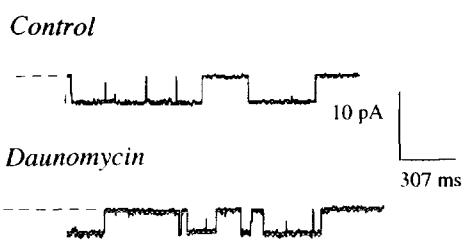

Figure 6. A 300 pS Channel in B30 Membrane Is Not Inhibited by Daunomycin (50 μM). The upper tracing shows activity of 300 pS channel in control, untreated membranes at a holding potential of +10 mV. Pretreatment of cells with 50μM daunomycin fails to induce any change in channel activity. The stippled line shows zero current level. Downward deflection is indicative of inward chloride current.

not inhibit channel activity. On the other hand, some findings reported in this paper are difficult to reconcile with Gill's hypothesis. For example, Gill (4) showed that inhibition of whole-cell chloride currents by transport substrates required ATP hydrolysis. However, our data indicates that channel inhibition by colchicine occurs in the nominal absence of ATP, in excised patches exposed to ATP-free solutions. Secondly, Gill (4) reported that channel block exhibited sidedness and vincristine, for example, only blocked channel activity when added to the cytosolic membrane surface. However, in our experiments, colchicine, produced similar inhibition of channel function when applied to either side. Likewise, verapamil induces block of the 40 pS channel when applied to either side of the membrane. Not only does verapamil (50 μM) cause channel block when applied to the cytosolic membrane surface as in the present experiments but Champigny et.al.[18] found that (±) verapamil (100 μM)and (-) desmethoxyverapamil (60 μM) induce a flickery-type block of this chloride channel when applied to outside-out patches. Hence, discrepancies exist between previous whole cell data and the present single channel studies which must be resolved prior to identification of the P-glycoprotein associated conductance path at the single channel level.

To conclude, our observations show that drugs known to stimulate P-glycoprotein ATPase activity act to modify chloride channel kinetics and support the suggestion that a single protein may assume both functions. However, it remains possible that this 40 pS channel is unrelated to P-glycoprotein except that it too is susceptible to modulation by a wide variety of compounds. In fact, this channel is known to be blocked by several different types of chloride channel blocker, including the anthracilic acid NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid) and the disulfonic stilbene, DNDS (4,4'-dinitro-stilbene-2,2'-disulfonic acid) (16,17). The structures of these compounds are quite different from those of the P-glycoprotein substrates discussed in this paper, however, they share some overall similarities in that both possess at least two aromatic rings and nitro groups. Finally, the most convincing data regarding the channel activity of P-glycoprotein will come from planar bilayer studies of the purified protein. Interestingly, preliminary studies of partially purified P-glycoprotein have revealed chloride-selective channel activity exhibiting similar properties to the channel discussed in this study, namely, an intermediate conductance of approximately 50 pS and an outwardly-rectifying current-voltage relationship [19].

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