Effects of steroids and verapamil on P-glycoprotein ATPase activity: progesterone, desoxycorticosterone, corticosterone and verapamil are mutually non-exclusive modulators

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P-glycoprotein (P-gp) is a membranous ATPase responsible for the multidrug resistance (MDR) phenotype. Using membrane vesicles prepared from the highly resistant cell line DC-3F/ADX we studied the influence on P-gp ATPase activity of four progesterone derivatives which specifically bind to P-gp and reverse MDR. Progesterone and desoxycorticosterone stimulate P-gp ATPase activity with, respectively, apparent concentrations giving half-maximal activation of 20–25 μ M and 40–50 μ M, and activation factors of 2.3 (at 100 μ M progesterone) and 1.8 (at 170 μ M desoxycorticosterone). Hydrocortisone above 100 μ M stimulates P-gp ATPase activity while corticosterone has no apparent stimulating effect. Our data are consistent with the location of the binding sites for the progesterone derivatives on the P-gp membranous domain. The effects of these steroids on

verapamil-stimulated P-gp ATPase activity support a non-competitive mechanism, i.e. the binding sites for verapamil and steroids are mutually non-exclusive for P-gp ATPase modulation. A similar non-competitive inhibition of progesterone-stimulated P-gp ATPase activity by desoxycorticosterone or by corticosterone leads to the conclusion that these steroids, although sharing related structures, have distinct modulating sites on P-gp. As expected from their mutually non-exclusive interactions on P-gp, progesterone and verapamil when mixed induce a synergistic modulation of P-gp ATPase activity. Since drug transport by P-gp is believed to be coupled to its ATPase activity, a corresponding synergistic effect of these two modulators for the inhibition of P-gp-mediated drug resistance can be expected.

INTRODUCTION

The multidrug resistance (MDR) phenotype gives some tumour cells the capability to survive at higher concentrations of various cytotoxic drugs than the corresponding wild-type sensitive cells can tolerate [1]. This can be observed *in vitro* with cultured cells [2] as well as *in vivo* in clinical tumours of human patients [3]. The MDR cells display cross-resistance patterns to various molecules sharing neither analogous structures nor similar toxicity mechanisms. The most typical and the most widely reported MDR phenotype is linked to the overexpression of a 170 kDa glycoprotein, P-glycoprotein (P-gp), in the plasma membrane of the resistant cells. P-gp is stated to be responsible for the ATP-dependent extrusion of the drugs out of the cytosol of the MDR cells [4]. A number of amphiphilic molecules have been found to reverse (at least partially) the MDR phenotype, and they were generally observed to be also transported by P-gp [5].

The P-gp primary structure displays two consensus sequences for intracytoplasmic nucleotide binding [4]. ATPase activity has been reported for P-gp in various experimental models: P-gp-containing membrane vesicles prepared from highly resistant cells [6,7], cells transfected by the *mdr* gene [8], immuno-adsorbed P-gp [9], detergent-extracted P-gp [10], and proteoliposomes obtained after reconstitution of partially [11,12] or highly purified P-gp [13,14]. The physiological significance of this basal activity is still unknown although several functional hypotheses have been proposed, for example a phospholipid translocase activity [15]. Depending on the experimental system considered, several drugs reported to be transported by P-gp induced various degrees of stimulation of P-gp ATPase activity. These observations, associated with experimental evidence of ATP-dependent drug

accumulation in P-gp-containing membrane vesicles [16–18], support the coupling between drug transport and ATP hydrolysis, which represents the currently most accepted working hypothesis for P-gp functioning [4]. However, the molecular mechanism governing the broad specificity of P-gp interactions with various drugs is still not understood, despite recent transport and photolabelling experiments reporting either competitive or non-competitive inhibition mechanisms [19–23]. In this context, P-gp ATPase activity measurements should provide another fruitful method for investigating drug transport and P-gp interaction mechanisms.

P-gp is expressed in various normal tissues [24], and among various physiological roles proposed it was suggested that some steroid hormones could be physiological substrates transported by P-gp [25–27]. This was confirmed by P-gp photoaffinity labelling experiments with progesterone and corticosterone [28,29], and by measurements of steroid transport out of MDR cells [29,30] or through a polarized epithelial monolayer of cells transfected by the P-gp gene [31]. Moreover, steroids displayed typical properties of MDR-reversing agents: increased vinblastine sensitivity of MDR cells [31–33], inhibition of P-gp-mediated *Vinca* alkaloid membrane transport [32–35], inhibition of P-gp labelling by a photoaffinity analogue of *Vinca* alkaloids [36], and inhibition of azidopine binding to P-gp [31,32,35].

We report here the influence on P-gp ATPase activity of four steroids derived from progesterone. For this study, we used P-gp-containing membrane vesicles purified from MDR cells, the highly resistant Chinese hamster lung fibroblasts DC-3F/ADX [37]. These vesicles were previously characterized for their ATPase activity in the absence of drugs ('basal ATPase activity') as well as in the presence of verapamil, a well-known MDR-

Abbreviations used: P-gp, P-glycoprotein; MDR, multidrug resistance; DTT, dithiothreitol.

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reversing agent which stimulates P-gp ATPase activity [7]. Our data show that: (i) progesterone is as effective as verapamil for stimulating P-gp ATPase activity; (ii) there is a clear correlation between decreasing hydrophobicity of the studied steroids and the increased steroid concentrations modulating the basal and stimulated P-gp ATPase; (iii) progesterone, desoxycorticosterone, corticosterone and verapamil are mutually non-exclusive modulators of P-gp ATPase activity, which implies that P-gp has at least four different and independent, but interacting, binding sites for these four compounds; (iv) mixtures of progesterone and verapamil display a synergistic effect on P-gp ATPase activity. These results were presented in part at the 39th Annual Meeting of the American Biophysical Society [38].

EXPERIMENTAL

Chemicals

Verapamil (hydrochloride), progesterone (pregn-4-ene-3,20-dione), desoxycorticosterone (21-hydroxyprogesterone), corticosterone (11 β ,21-dihydroxyprogesterone), hydrocortisone (11 β ,17 α ,21-trihydroxyprogesterone), ouabain and EGTA were from Sigma; sodium azide was from Merck and pyruvate kinase and lactate dehydrogenase were from Boehringer Mannheim. All other products were reagent grade.

Cells

Spontaneously transformed Chinese hamster DC-3F lung fibroblasts and their MDR-derived, actinomycin D-selected, DC-3F/ADX cells were cultured, harvested and frozen as previously described [7].

Preparation of total membrane vesicles

Membrane vesicles were prepared from DC-3F or DC-3F/ADX cells as previously described [7]. Frozen cells were thawed, washed twice in PBS and collected by centrifugation (1000 *g*, 5 min, 4 °C). They were then suspended in a hypotonic lysis buffer [10 mM Tris/HCl, pH 7.8, at 20 °C, 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 1 mM EGTA] and allowed to swell for 20 min at 4 °C. Swollen cells were disrupted by sonication for 10 s at 20 % maximum power (Sonicator W-225 R, Heat System-Ultrasonics) and the resulting homogenate was centrifuged (1400 *g*, 10 min, 4 °C). The supernatant was then layered on a 46 % (w/w) sucrose cushion in lysis buffer and centrifuged (7000 *g*, 20 min, 4 °C). The layer at the sucrose interface was removed, diluted twice with lysis buffer and sedimented (135000 *g*, 15 min, 4 °C). The pellet of membrane vesicles was washed twice in lysis buffer and finally resuspended in lysis buffer supplemented with 100 mM NaCl, at a total membrane protein concentration of 2.2–3.5 mg/ml. Protein concentrations were determined by Bradford's method using the Bio-Rad protein assay kit, with BSA as a standard.

ATPase activity measurement

ATPase activities were measured at 37 °C with a coupled enzyme assay by continuous monitoring of absorbance at 340 nm, as described previously [7]. The reaction medium was composed of 10 mM Tris/HCl, pH 7.8 (at 20 °C), 100 mM NaCl, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, and 1 mM MgATP (except when otherwise stated), and was supplemented with 1 mM phosphoenolpyruvate, 0.5 mM NADH, 0.1 mg/ml lactate dehydrogenase and 0.1 mg/ml pyruvate kinase in order to maintain MgATP concentration constant in the medium. Some membrane

ATPases were inhibited by addition of 10 mM sodium azide, 0.5 mM ouabain and 1 mM EGTA. To limit the dilution effect in covering the selected steroid, verapamil or MgATP concentration ranges, two stock solutions of each compound, whose concentrations differed by an appropriate factor, were prepared. The concentrated stock solutions of the steroids tested were dissolved in ethanol, and that of verapamil was dissolved in DMSO, which allowed a satisfactory solubility of the highest modulator concentrations tested. Since absorbance spectra of steroid solutions are sensitive to steroid solubility because of turbidity changes, steroid solubility was verified by control measurements of their absorbance spectra, which showed no turbidity change within the concentration ranges tested in the ATPase activity measurements (results not shown). Final ethanol or DMSO concentration in the assay medium never exceeded 4 % (v/v), which had no effect on the measured ATPase activity. Successive additions of the compound whose concentration influence was being studied were made to the same optical cuvette under continuous stirring. Dilution effects were taken into account for final ATPase activity calculations. The data presented are representative curves of two to five identical experiments, performed using four different membrane vesicle batches prepared from three different large-scale cell culture harvestings. For the MgATP concentration-dependence curves, data analysis was performed by non-linear regression fitting (Sigma Plot software), according to the Hill equation to derive the enzymic parameters K_m , V_{max} and h .

The ATPase activity measurement method using a coupled enzyme assay has some practical advantages over radioactive or colorimetric methods for determining inorganic phosphate production, such as rapidity, reliability and an unchanging MgATP concentration in the medium. It also provides continuous measurements of different points of a concentration-dependence curve by successive additions of the molecule being tested to the reaction medium in the same absorbance cuvette. Thus, this procedure gives good confidence in the overall shape of these concentration-dependence curves, allowing reasonably accurate determinations of their physico-chemical parameters, such as apparent affinity and co-operativity.

RESULTS

Effects of progesterone and verapamil on basal P-gp ATPase activity

The ATPase activity of a suspension of membrane vesicles prepared from MDR cells was measured in a medium containing 1 mM MgATP and supplemented with three ATPase inhibitors (sodium azide, ouabain and EGTA) which are, respectively, specific for mitochondrial F-ATPases, Na⁺/K⁺-ATPase and Ca²⁺-ATPases (see the Experimental section). The ATPase activity determined under these conditions, 0.21 ± 0.05 μ mol/mg of membrane protein per min ($n = 5$), is defined as the 'basal' P-gp ATPase activity and represents around 50 % of ATPase activity measured in the absence of inhibitors. It has been shown that this basal activity can be attributed to P-gp, which represents about 15 % (w/w) of the total membrane proteins [7]. The specific P-gp ATPase activity is then 1.4 ± 0.3 μ mol/mg of P-gp per min ($n = 5$).

Figure 1(A) shows the effect of increasing progesterone concentrations from 0.4 to 350 μ M on basal P-gp ATPase activity. For the lower progesterone concentrations, P-gp ATPase activity progressively increases and reaches a maximal value of 0.49 ± 0.08 μ mol/mg of membrane protein per min ($n = 5$) at 100 μ M progesterone. The ratio of maximal to basal P-gp ATPase activity, that is the apparent activation factor, is 2.3 ± 0.3 ($n = 5$), and the progesterone concentration inducing the apparent half-

Figure 1 Effect of progesterone on P-gp ATPase activity

MgATP hydrolysis rates are measured using a coupled enzyme assay, with 10 mM sodium azide, 0.5 mM ouabain and 1 mM EGTA. (A) With 1 mM MgATP, increasing concentrations of progesterone are obtained by successive additions to the same optical cuvette, which contains either 14 μg/ml membrane proteins from DC-3F/ADX cells (■) or 50 μg/ml membrane proteins from DC-3F cells (□). (B) With 100 μM progesterone (▼), with 10 μM verapamil (●), or without any modulator (open symbols), increasing concentrations of MgATP are obtained by successive additions to the same cuvette, which contains either 20 μg/ml membrane proteins from DC-3F/ADX cells (closed symbols and diamonds) or 100 μg/ml membrane proteins from DC-3F cells (□). Curves are fitted according to the Hill equation and give the following values for Hill coefficient (*h*): ◇, *h* = 1.00 ± 0.10 and *h* = 0.97 ± 0.14; ●, *h* = 1.06 ± 0.06; ▼, *h* = 1.02 ± 0.09.

maximal activation is 20–25 μM. For progesterone concentrations above 100 μM, P-gp ATPase activity decreases sharply. Membrane vesicles devoid of P-gp, prepared from the parental sensitive cell line, display a very low ATPase activity which is not altered by adding progesterone (Figure 1A). Thus, the bell-shape dependence of ATPase activity on progesterone concentration observed in the membrane vesicles prepared from MDR cells appears to be related to P-gp. Stimulation of P-gp ATPase activity by progesterone has been reported recently for membrane fractions obtained from cells transfected by MDR1 cDNA [39] and from purified P-gp reconstituted into proteoliposomes [14], showing a similar bell-shape dependence and with approximately the same progesterone concentration inducing maximal stimulation of P-gp ATPase activity. This agreement between the data obtained from different P-gp-containing membranes

reinforces confidence in this ATPase activity dependence on progesterone being attributable to P-gp.

The bell-shape dependence of P-gp ATPase on progesterone concentration can be compared with the other bell-shape dependence that was previously observed with the well-known MDR-reversing agent verapamil. In contrast to progesterone, the verapamil curve displays a smoother maximum; verapamil concentrations inducing half-maximal and maximal activation of ATPase activity were, respectively, 1–1.5 μM and 10–20 μM, with an activation factor of $1.70 \pm 0.25 \mu\text{M}$ (*n* = 13) ([7], and see the control curves in Figures 3 and 4, closed circles). This functional characteristic of P-gp has been confirmed by several studies on different experimental membranous systems: Sf9 insect cells transfected by MDR1 cDNA [8], native vesicle membranes from MDR Chinese hamster ovary cells [6], and proteoliposomes containing partially purified P-gp [12]. These bell-shape dependences of the P-gp ATPase could be reminiscent of a typical membrane-active transporter whose transport sites alternate between high- and low-affinity states [40]. However, the inhibition phase observed at high concentrations of these two amphiphilic agents could also be attributed to non-specific membrane perturbation.

Figure 1(B) represents the P-gp ATPase activity variations when the MgATP concentration was increased from 0.020 to 10 mM. In the absence of any drug, the dependence of basal P-gp ATPase activity on MgATP concentration is clearly fitted to a Michaelian curve, with $K_m \approx 670 \pm 140 \mu\text{M}$. With 100 μM progesterone, P-gp ATPase activity also exhibits Michaelian behaviour, with $K_m \approx 400 \pm 50 \mu\text{M}$. This is practically the same MgATP concentration dependence as with 10 μM verapamil ($K_m \approx 405 \pm 30 \mu\text{M}$). Thus, the P-gp ATPase activation observed at the maximal verapamil or progesterone activation concentrations is not a consequence of large variations in the corresponding K_m values for MgATP.

Comparison of effects of different steroids on basal P-gp ATPase activity

Figure 2 shows the effects on P-gp ATPase activity of increasing concentrations of various steroids: desoxycorticosterone, corticosterone and hydrocortisone, whose chemical structures are derived from progesterone by increasing the number of hydroxyl groups on the steroid moiety. As with progesterone, the effect of increasing desoxycorticosterone concentrations on P-gp ATPase activity follows a bell-shape dependence, but it differs by a higher apparent half-maximal activation concentration, $\approx 40\text{--}50 \mu\text{M}$, and a slightly lower apparent activation factor, $\approx 1.8 \pm 0.6$ (*n* = 3). In contrast, corticosterone has only an inhibitory effect on P-gp ATPase activity, and hydrocortisone only induces a progressive stimulation of P-gp ATPase activity at concentrations above $\approx 200 \mu\text{M}$ (Figure 2). Control experiments using a suspension of membrane vesicles devoid of P-gp display no significant effects of these three steroids on the observed ATPase activity (Figure 2). This eliminates the possibility that a non-specific effect of these steroids on other membrane ATPases could be responsible for the observed steroid-induced ATPase stimulation in P-gp-containing vesicles. It thus appears that increasing the hydrophilicity of the tested steroids, by increasing the number of hydroxyl groups on the polycyclic moiety [41], leads to complex effects on P-gp ATPase activity.

Comparison of effects of different steroids on verapamil-stimulated P-gp ATPase activity

As corticosterone has no activating effect on basal P-gp ATPase activity, it is expected that any competing effect of corticosterone

Figure 2 Effect of a series of hydroxylated derivatives of progesterone on P-gp ATPase activity

MgATP hydrolysis rates are measured in a medium composed as in Figure 1 and supplemented with 1 mM MgATP. Increasing concentrations of each steroid are obtained by successive additions to the same cuvette, which contains either 17 $\mu\text{g}/\text{ml}$ membrane proteins from DC-3F/ADX cells (closed symbols) or 45 $\mu\text{g}/\text{ml}$ membrane proteins from DC-3F cells (open symbols); $\circ, \bullet$: desoxycorticosterone; $\triangle, \Delta$: corticosterone; ∇, ∇ : hydrocortisone.

Figure 3 Effect of increasing corticosterone concentrations on verapamil-induced ATPase activity of P-gp

MgATP hydrolysis rates are measured as in Figure 2. Increasing concentrations of verapamil are obtained by successive additions to the same cuvette, which contains 30 $\mu\text{g}/\text{ml}$ membrane proteins from DC-3F/ADX cells, of 0 μM ($\bullet$), 10 μM ($\circ$), 50 μM ($\square$), 200 μM ($\triangle$) or 800 μM (∇) corticosterone. Without verapamil, the ATPase activities measured with increasing corticosterone concentrations are normalized with respect to the basal activity (set at 100%) obtained before the addition of either drug, which was 150–170 nmol/mg per min.

on verapamil-stimulated P-gp ATPase activity should be clearly shown in this case. The verapamil concentration-dependence of P-gp ATPase activity with various initial corticosterone concentrations in the medium was measured. Without corticosterone (Figure 3, $\bullet$), verapamil concentrations inducing half-maximal (2 μM) and maximal activation of ATPase activity are consistent with previous measurements [7]. As the initial corticosterone concentration increased from 10 to 200 μM , the verapamil-

Figure 4 Effect of increasing progesterone or desoxycorticosterone concentrations on verapamil-induced ATPase activity of P-gp

MgATP hydrolysis rates are measured as in Figure 2. (A) Increasing concentrations of verapamil are obtained by successive additions to the same cuvette, which contains 18 $\mu\text{g}/\text{ml}$ membrane proteins from DC-3F/ADX cells, of 0 μM ($\bullet$), 4 μM ($\circ$), 20 μM ($\square$) or 100 μM ($\triangle$) progesterone. Without verapamil, the ATPase activities measured with increasing progesterone concentrations are normalized with respect to the basal activity (set at 100%) obtained before the addition of either drug, which was 250–380 nmol/mg per min. (B) Increasing concentrations of verapamil are obtained by successive additions to the same cuvette, which contains 32 $\mu\text{g}/\text{ml}$ membrane proteins from DC-3F/ADX cells, of 0 μM ($\bullet$), 10 μM ($\circ$), 40 μM ($\square$) or 170 μM ($\triangle$) desoxycorticosterone. Without verapamil, the ATPase activities measured with increasing desoxycorticosterone concentrations are normalized with respect to the basal activity (set at 100%) obtained before the addition of either drug, which was 120–160 nmol/mg of membrane protein per min.

stimulated P-gp ATPase activity curves progressively display decreasing activation factors, while the half-maximal activation verapamil concentrations remain fairly constant (≈ 2 –3 μM) (Figure 3, open symbols). These data show that corticosterone appears to be a non-competitive inhibitor of verapamil-stimulated P-gp ATPase activity.

We have observed that verapamil and progesterone display comparable activation factors for P-gp ATPase activity, although they have very different chemical structures. This apparent broad specificity of P-gp ATPase modulation by these two molecules was investigated by studying the influence of combinations of these two modulators on P-gp ATPase activity. After an initial

addition of 4 μM progesterone, verapamil induces further ATPase activation following a bell-shape curve with an amplitude similar to that observed without progesterone (Figure 4A, circles). However, with 20 μM progesterone (concentration inducing half-maximal activation, see Figure 1A), increasing verapamil concentrations do not induce a further activation of P-gp ATPase activity (Figure 4A, squares). Moreover, with 100 μM progesterone (concentration inducing maximal activation, see Figure 1A), increasing verapamil concentrations induce a monotonic inhibition of P-gp ATPase activity even at submicromolar concentrations (Figure 4A, triangles). Thus, additive effects are not observed for P-gp ATPase activation by these two modulators, as would be expected if two or more P-gp isoforms were present, such as those characterized by their different transport properties for verapamil and progesterone [33]. Rather, since at 10–20 μM verapamil all the P-gp ATPase activity values observed with increasing progesterone concentrations are lower than the value determined without progesterone, the data suggest a non-competitive mechanism for the inhibition of verapamil-stimulated ATPase activity. Moreover, the observed behaviour is not compatible with a progesterone/verapamil competition mechanism for P-gp ATPase activation, since in this case one would expect the maximal activation (with respect to the basal ATPase activity) induced by verapamil in the presence of progesterone to be comparable with the one obtained in the absence of progesterone, but with higher concentrations necessary to displace progesterone initially bound to activate P-gp.

In comparable experiments, we have studied the verapamil concentration-dependence of P-gp ATPase with various desoxycorticosterone concentrations (Figure 4B). These data show the same behaviour as with progesterone in Figure 4(A), i.e. progressive decrease of the activation induced by verapamil (open symbols). In particular, with the desoxycorticosterone concentration that induced maximal activation of basal P-gp ATPase activity (170 μM , see Figure 2), a monotonic inhibition is observed with increasing verapamil concentrations (triangles in Figure 4B). These results also indicate a non-competitive inhibition of the verapamil-stimulated P-gp ATPase activity. However, on studying the verapamil concentration-dependence of P-gp ATPase activity with hydrocortisone concentrations up to 530 μM , we did not observe any significant effect of this steroid (results not shown).

Experimental evidence for a synergistic effect of progesterone and verapamil on P-gp ATPase activity

To verify that the influence of verapamil and progesterone on P-gp ATPase activity is independent of the addition order of the two modulators, we performed a 'symmetrical' experiment in which verapamil was added first at various concentrations (Figure 5). We obtained an inhibition pattern similar to the one represented in Figure 4(A): the higher the initial activation of ATPase by verapamil, the lower the P-gp ATPase activity measured at 100 μM progesterone (open symbols). Moreover, at saturating concentrations of progesterone (100 μM) and verapamil (10–20 μM) together, the final P-gp ATPase activity was very similar in both experiments (compare Figures 4A and 5). These results indicate mutual non-competitive inhibitory effects of verapamil and progesterone for P-gp ATPase stimulation, without irreversible alteration of P-gp function.

To characterize further the relationship between the effects of verapamil and progesterone on modulating P-gp ATPase, we measured the variations of the basal P-gp ATPase activity with increasing concentrations of various mixtures of verapamil and progesterone in molar ratios of 1/6, 1/12 (ratio of their respective

Figure 5 Effect of increasing verapamil concentrations on progesterone-induced ATPase activity of P-gp

MgATP hydrolysis rates are measured as in Figure 2. Increasing concentrations of progesterone are obtained by successive additions to the same cuvette, which contains 30 $\mu\text{g}/\text{ml}$ membrane proteins from DC-3F/ADX cells, of 0 μM (●), 0.3 μM (○), 3 μM (□) or 30 μM (△) verapamil. Without progesterone, the ATPase activities measured in the presence of increasing verapamil concentrations are normalized with respect to the basal activity (set at 100%) obtained before the addition of either drug, which was 90–110 nmol/mg of membrane protein per min.

half-maximal activation concentrations) and 1/24. We observed in all cases a bell-shape curve (results not shown). In the isobole representations for the half-maximal activation, as well as for the maximal activation of P-gp ATPase, all the tested mixtures of verapamil and progesterone are clearly below the additivity line (results not shown). According to Berenbaum [42], this observation of a synergistic effect for these two modulators is consistent with their non-competitive effects on P-gp ATPase.

Mutual effects of various combinations of two steroids on P-gp ATPase activity

We concluded from our enzymic experiments that structurally unrelated compounds like verapamil and progesterone derivatives, which all modulate P-gp ATPase activity, do not compete for P-gp stimulation. However, it remains to be seen whether closely structurally related compounds like progesterone, corticosterone and desoxycorticosterone compete with each other. The effects of progesterone on P-gp ATPase activity in the presence of increasing concentrations of either corticosterone or desoxycorticosterone are represented, respectively, in Figures 6(A) and 6(B). With corticosterone, which alone does not activate basal P-gp ATPase activity, we observe that increasing initial corticosterone concentrations in the medium progressively inhibit activation of P-gp ATPase activity by progesterone without any significant shift of the maximal activation value towards higher progesterone concentrations. This behaviour, similar to that represented in Figure 3, is clearly not compatible with a competition process and rather favours a non-competitive inhibition mechanism. In addition, corticosterone has a similar inhibitory effect on the ATPase activity stimulated by desoxycorticosterone (results not shown).

With desoxycorticosterone (Figure 6B), which alone stimulates basal P-gp ATPase activity, increasing its concentration in the medium induces a progressive inhibition of the stimulation of P-gp ATPase activity by progesterone, similar to that observed in

Figure 6 Effect of increasing corticosterone or desoxycorticosterone concentrations on progesterone-induced ATPase activity of P-gp

MgATP hydrolysis rates are measured as in Figure 2. (A) Increasing concentrations of progesterone are obtained by successive additions to the same cuvette, which contains 17 μg/ml membrane proteins from DC-3F/ADX cells, of 0 μM (●), 3 μM (○), 30 μM (□), 250 μM (△) or 800 μM (▽) corticosterone. Without progesterone, the ATPase activities measured with increasing corticosterone concentrations are normalized with respect to the basal activity (set at 100%) obtained before the addition of either drug, which was 110–230 nmol/mg per min. (B) Increasing concentrations of progesterone are obtained by successive additions to the same cuvette, which contains 30 μg/ml membrane proteins from DC-3F/ADX cells, of 0 μM (●), 30 μM (○) or 170 μM (□) desoxycorticosterone. Without progesterone, the ATPase activities measured with increasing desoxycorticosterone concentrations are normalized with respect to the basal activity (set at 100%) obtained before the addition of either drug, which was 90–100 nmol/mg of membrane protein per min.

Figure 4(B). In particular, at 100 μM progesterone and 170 μM desoxycorticosterone, the ATPase activity value is lower than that observed without desoxycorticosterone, in agreement with non-competitive effects of these two steroids on P-gp ATPase stimulation. Thus, according to these experiments, progesterone, corticosterone and desoxycorticosterone, although sharing a fairly similar chemical structure, are unable to compete with each other for P-gp activation.

Comparison of the ATPase activity curves in Figures 4(A), 4(B), 5 and 6(B) shows in each case that all the curves cross each other at a constant point, independent of the initial modulator concentration in the activity measurements. This kind of 'isosbestic' point can obviously be observed only when testing

two modulators which are activators of basal ATPase activity (this is not observed with corticosterone, see Figure 3). Enzyme kinetics calculations based on Michaelis–Menten equations, assuming a simple model with one substrate (MgATP) whose catalytic reaction can be activated by two mutually non-exclusive ligands, show that such an isosbestic point does exist (calculations not presented). Within approximations that are reasonable from our experimental data (the two modulators have the same activation factor, the activity of the ternary complex with the two modulators simultaneously bound is the same as the basal activity, the K_m constants for MgATP hydrolysis are the same with or without either modulator, the binding affinity for either modulator is independent of the binding of the other modulator), we found that the isosbestic point abscissa is equal to the half-maximal activation concentration of the modulator whose variable concentration defines the graph abscissa. This is in agreement with experimental data (Figures 4A, 4B, 5 and 6B) and further supports the mutually non-competitive effects of two activators on P-gp.

DISCUSSION

The dependence of ATP hydrolysis on MgATP concentration in the presence of 100 μM progesterone displays K_m and V_{max} values significantly different from those determined for the basal P-gp ATPase activity (i.e. without modulator), but very similar to what is observed with 10 μM verapamil. This suggests that progesterone and verapamil modulate the P-gp enzymic state in the same way. Moreover, the MgATP concentration dependence is not co-operative in the absence or presence of either of these two modulators, indicating that either the two ATP-binding sites of P-gp hydrolyse MgATP independently, or only one is capable of hydrolysing MgATP. This last possibility can be rejected since Loo and Clarke [43] demonstrated that each half of human P-gp, when expressed separately, exhibits basal ATPase activity. Moreover, it was shown in the same work that interaction between both halves of P-gp is required to exhibit drug-stimulated ATPase activity. To reconcile all these results, we can assume that only one ATP-binding site is involved in the drug-stimulated ATPase activity. This is strengthened by the fact that 100% of the verapamil-stimulated P-gp ATPase activity is inhibited when only 1 mol of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole per mol of P-gp is bound to the C-terminal part of P-gp [6,44]. The ATP-binding site located in the C-terminal half of P-gp would thus be the only one directly involved in the drug-stimulated activity. However, integrity of the two ATP-binding sites is necessary to confer drug transport capacity as demonstrated by discrete mutations introduced in the two nucleotide-binding sites [45]. All the data taken together suggest that the two nucleotide-binding sites on P-gp have distinct functions. Recently, Carson et al. [46] reached a similar conclusion concerning the two nucleotide-binding domains of the cystic fibrosis transmembrane conductance regulator which, like P-gp, belongs to the ATP-Binding-Cassette superfamily of membrane transporters.

The aim of this work was to address by an enzymic approach the relationship between the modulating sites on P-gp of molecules known to reverse the MDR phenotype and to interact with P-gp. We used chemically unrelated molecules (verapamil and progesterone) as well as a series of closely structurally related steroids in order to conveniently investigate the apparent broad specificity of P-gp for the various agents which modulate its function. Since these molecules are amphiphilic or hydrophobic, we have to consider possible non-specific effects on P-gp function due to a global membrane perturbation. The possibility that such non-specific effects could be responsible for the observed increases

in P-gp ATPase activity induced by verapamil or steroids can be ruled out for at least the following reasons: (i) control experiments on membrane vesicles prepared from parental sensitive DC-3F cells (devoid of P-gp) show, for the same ranges of concentrations of verapamil or steroids, neither activation of H^+ -ATPases, Na^+/K^+ -ATPase and Ca^{2+} -ATPases, considered as models of membrane ATPases which could be sensitive to membrane perturbations (results not shown), nor effects on the global MgATPase activity, which indicates that ATPase activation observed in membranes from MDR cells is due to P-gp; (ii) vesicle permeability changes exposing new MgATP hydrolysis sites would induce similar MgATPase activation for control membrane vesicles as well as for P-gp-containing membrane vesicles and for all tested amphiphilic compounds, which is not observed; (iii) typical membrane perturbations by non-solubilizing concentrations of various detergents have always been shown to inhibit, not to activate, P-gp ATPase activity [47]; (iv) after CHAPS-induced solubilization, P-gp still displays ATPase activation for comparable verapamil or progesterone concentrations [10]. Moreover, it has been shown by photolabelling experiments that the compounds tested here for their influence on P-gp ATPase activity are able to bind specifically to P-gp [28,29,48,49]. It is thus highly probable that the stimulatory effects on P-gp ATPase activity induced by these compounds are related to their binding on P-gp. On the other hand, the inhibition of P-gp ATPase observed with the higher concentrations of verapamil and steroids could well be due to a global membrane-perturbing effect. For this reason, we always tested on the drug-stimulated P-gp ATPase activity the influence of modulators at concentrations within ranges activating the basal ATPase in order to obtain specific binding of these modulators on P-gp.

The binding sites of the various steroids tested can, in a first approach, be described by the concentrations that elicit half-maximal ATPase activation, which can only be considered as a gross evaluation of their apparent affinity. These values appear to increase in the same order as the increasing hydroxylation of the steroid molecules, i.e. $\approx 25 \mu M$ for progesterone, $40\text{--}50 \mu M$ for desoxycorticosterone, and $> 200 \mu M$ for hydrocortisone. Corticosterone is not included in this classification because it does not induce observable activation of basal ATPase activity despite the fact that it binds to P-gp [29]. However, corticosterone binding to P-gp can be shown by its non-competitive inhibitory effect on verapamil-stimulated ATPase activity. It is then worth comparing the relative efficiency of the four steroids for half-inhibition of the maximal verapamil-stimulated ATPase activity: i.e. roughly $20 \mu M$ for progesterone, $40 \mu M$ for desoxycorticosterone and $50 \mu M$ for corticosterone. No effect was observed for hydrocortisone up to $530 \mu M$. Thus, the relative modulation efficiency of these steroids is the same whether considering either the basal or the verapamil-stimulated P-gp ATPase activity. Moreover, in the case of progesterone and desoxycorticosterone, modulation of basal and verapamil-stimulated ATPase activity seems to depend on the same sites. In addition, progesterone- and verapamil-stimulated ATPase activities are inhibited by comparable concentrations of either desoxycorticosterone or corticosterone, which also indicates similarity between these modulating sites. Moreover, progesterone-stimulated ATPase activity is inhibited by verapamil concentrations comparable with those which activate basal P-gp ATPase activity. Altogether, these observations indicate that for each tested modulator the same specific binding sites could be responsible for the modulation of both basal and drug-stimulated P-gp ATPase activity. The working hypothesis of enzymic coupling between drug transport and drug-stimulated ATPase activity would imply similarity of sites stimulating ATP hydrolysis and transport sites

on P-gp. We are thus led to a simple model of P-gp possessing, for each drug/modulator, a 'multi-function site' responsible for the stimulation of ATPase activity and transport as well as the simultaneous inhibition of ATPase activity and transport stimulated by another drug/modulator. In this context, a previously reported set of inhibition constant values for progesterone, desoxycorticosterone and hydrocortisone on vincristine accumulation into P-gp-containing membrane vesicles is consistent with our apparent affinities drawn from P-gp ATPase activity measurements [34]. There is also a good agreement between our data on the inhibition of progesterone-stimulated ATPase by verapamil and the inhibition of vinblastine transport by similar verapamil concentrations [16]. Moreover, the hydrophobicity increase of the four studied steroids is directly correlated with their inhibitory efficacy on both vinblastine efflux out of MDR cells [32] and verapamil-stimulated P-gp ATPase activity (this work).

Since the influence on P-gp ATPase activity of all the studied modulators is mediated through their specific binding to P-gp, we investigated the possibility that they might share a common site, i.e. we wondered whether competition effects could be observed through their mutual influence on P-gp ATPase activity. Thus each steroid was combined either with a different steroid or with verapamil to investigate their effect on P-gp ATPase activity. Our experimental results show that the mutual inhibition by verapamil and steroids of P-gp ATPase can be described by non-competitive mechanisms. Thus all these modulators can bind simultaneously on physically distinct binding sites on P-gp. Such a non-competitive mechanism between structurally unrelated compounds has been reported for inhibition of azidopine binding on plasma membranes of MDR cells by vinblastine or cyclosporin A [22], and for inhibition by verapamil of tetrahydropyranlyl-adriamycin efflux by living MDR cells [23]. Another indication of specific distinct binding sites for various drugs has been recently suggested by Rao [50], who reported that valine substitution at amino acid 185 in P-gp alters the colchicine- and verapamil-binding sites but not the vinblastine-binding sites. Alternatively, competitive inhibition has been reported of *Vinca* alkaloid transport by P-gp-containing membrane vesicles [19–21]. Thus, depending on the drugs considered, P-gp function modulation could involve competitive as well as non-competitive mechanisms.

Unexpectedly, although the steroids tested are structurally related molecules, they influence drug-stimulated P-gp ATPase activity by non-competitive inhibition mechanisms, which demonstrates that these steroids modulate P-gp ATPase activity through specific distinct sites. Moreover, the more hydroxylated progesterone derivatives, which have the lowest membrane-aqueous-solution partition coefficients, display the lowest apparent affinities. This suggests that the distinct binding sites for the various modulators are located in P-gp membranous domain(s) in agreement with the 'flippase' model, where P-gp pumps the drugs from the core of the phospholipidic membrane, rather than the 'pump' model where drugs interact with P-gp from the aqueous phase [51,52]. In particular, this could explain the high sensitivity of drug-stimulated P-gp ATPase activity to the nature of lipidic environment as recently reported [53]. A somewhat analogous situation was recently proposed for the interaction with sarcoplasmic reticulum Ca^{2+} -ATPase of diethylstilbestrol and related hydrophobic compounds [54], and of halothane and various halogenated anaesthetics [55].

The hypothesis of direct coupling between drug-stimulated P-gp ATPase activity and drug transport allows us to derive some functional consequences from our enzymic data. First, since the simultaneous binding of two non-exclusive modulators stimulates

P-gp ATPase activity, they could be simultaneously transported by P-gp. However, compared with their intrinsic ATPase stimulation, their simultaneous binding results in significant inhibition of the modulator-stimulated P-gp ATPase activity. This should imply a decreased transport rate of both modulators, consistent with an energetically unfavourable situation with respect to separate transport of each modulator coupled with ATP hydrolysis. Secondly, the synergistic effect on the half-maximal activation concentration for the modulation of P-gp ATPase activity by two mutually non-exclusive modulators like verapamil and progesterone suggests a parallel synergistic effect of these compounds on inhibition of drug transport by P-gp. This would allow induction of a comparable inhibitory effect with lower concentrations of associated mutually non-exclusive modulators than if they were used separately. This might explain the effect of the combination of low concentrations of various lipophilic MDR-reversing drugs in increasing accumulation of actinomycin D in MDR cells [56].

In conclusion, the picture that emerges from our results is that P-gp possesses multiple binding sites for various hydrophobic compounds, which remain to be further characterized for their mechanistic, physiological and pharmacological properties. A first step to this goal would be a classification of the various molecules interacting with P-gp according to their mutually exclusive or non-exclusive binding properties, which would provide a rationale for the choice of MDR-reversing mixtures. Obviously, this could have consequences for future clinical trials aimed at overcoming resistance to chemotherapeutic agents.

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