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Modulatory Effects of Natural Curcuminoids on P-Glycoprotein ATPase of Insecticide-Resistant Pest *Helicoverpa armigera* (Lepidopetera: Noctüidae)

Ravindra M. Aurade · Senigala K. Jayalakshmi · Kuruba Sreeramulu

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Abstract Three major curcuminoids (I, II and III) were purified from turmeric and tested for their ability to modulate the function of P-glycoprotein ATPase of the insecticide-resistant pest *Helicoverpa armigera* (Ha-Pgp). The curcumin mixture inhibited the activity of Ha-Pgp ATPase by 80–90% at 100 μM concentration. Along with curcuminoids I, II and III, it inhibited the verapamil- and ethylparaoxon-stimulated Ha-Pgp ATPase activity. Curcuminoid binding was quantitated by quenching the intrinsic Trp fluorescence of purified Ha-Pgp ATPase. Transport was monitored in proteoliposomes containing Ha-Pgp ATPase using the high-affinity fluorescent substrate tetramethylrosamine (TMR) in real time. Addition of the curcuminoid mixture collapsed the TMR concentration gradient generated by Ha-Pgp ATPase. Inhibition studies on Ha-Pgp ATPase activity are important to develop strategies to overcome insecticide resistance in this pest.

Keywords *Helicoverpa armigera* · P-glycoprotein) · Curcuminoid · Proteoliposome · Tryptophan quenching · Drug transport

Introduction

The bollworm, *Helicoverpa armigera* (Lepidoptera: Noctüidae), is a polyphagous pest. It is ubiquitous all over the world and damages crops worth about US $1 billion in India alone. It has developed resistance to virtually every insecticide class that has been applied to it (Armes et al. 1992; Kranthi et al. 2001; Srinivas et al. 2004, 2006). Insects that show resistance to one insecticide generally develop resistance to another class of insecticides, a phenomenon often referred to as "cross-resistance." It resembles multidrug resistance (MDR) whereby resistance to one drug is accompanied by simultaneous resistance to a variety of structurally unrelated compounds (Lanning et al. 1996).

MDR in mammalian organisms has been associated with overexpression of P-glycoprotein (Pgp), which is a 170-kDa plasma membrane protein that belongs to the ATP-binding cassette (ABC) superfamily (Leslie et al. 2005). ABC superfamily proteins function as ATP-coupled import or export pumps (Dassa and Bouige 2001; Jones and George 2004; Locher 2004), and several of them that confer protection against the toxicity of various environmental xenobiotics have been reported (Leslie et al. 2005; Abu-Qare et al. 2003). In general, these proteins comprise the nucleotide-binding domains and are highly conserved throughout the protein family. It has also been proposed that Pgp acts as a hydrophobic vacuum cleaner (Gottesman and Pastan 1993), removing drugs from the plasma membrane rather than the aqueous phase. Pgp-mediated MDR is thought to be an important cause of failure of cancer chemotherapy. Pgp displays high levels of constitutive ATPase activity, which may be stimulated or inhibited by the addition of drug substrates (Sharom et al. 1995; Sharom 1997).

Earlier, we had detected that Pgp of *H. armigera* (Ha-Pgp) ATPase reconstituted into proteoliposomes and found that some insecticides stimulated the ATPase activity (Aurade et al. 2006). This protein was purified, characterized and found to be a 150-kDa glycoprotein displaying ATPase

R. M. Aurade · K. Sreeramulu (✉)
Department of Biochemistry, Gulbarga University, Gulbarga 585 106, Karnataka, India
e-mail: ksramu@rediffmail.com

S. K. Jayalakshmi
Agricultural Research Station, University of Agricultural Sciences, Raichur, Gulbarga 585 103, India

activity. This enzyme was partially sequenced and found to be homologous to conserved sequences of mammalian Pgp (Aurade et al. 2010). Similar ABC proteins have been implicated in the resistance of many organisms to a vast and chemically diverse range of toxic molecules, and this type of resistance has occurred throughout evolution (Prasad et al. 1996; Dassa and Bouige 2001). The role of Pgp-like proteins in insects merits study because such transporters may contribute to insecticide resistance.

The inhibiting properties of several chemicals on Pgp activity have been investigated. Verapamil, a calcium channel–blocking agent, was the first drug described as an inhibitor (Tsuruo et al. 1981). After this, second- and third-generation compounds have been studied for their inhibitory effect on Pgp (Sikic 1999; Twentyman and Bleehen 1991; Mayer et al. 1997). However, specific inhibitors of Pgp are still under development. Plants offer inexpensive and plentiful phytochemicals. Curcuminoids are natural phenolic coloring compounds found in the rhizomes of *Curcuma longa* Linn., commonly known as turmeric. Three main curcuminoids have been isolated and characterized from turmeric—curcumin I, II and III (Ramsewak et al. 2000; Chearwae et al. 2006). Curcumin exhibits ovicidal action against *H. armigera* eggs (Solsoloy et al. 1991), insecticidal activity against *Aedes aegyptii* (L) (Roth et al. 1998) and insect growth inhibitory activity against *Schistocerca gregaria* and *Dysdercus koenigii* (Hemanta et al. 2000). Curcumin inhibited human Pgp ATPase activity and drug transport in the drug-resistant cell line KB-V1 (Chearwae et al. 2004). Curcumin inhibits the ABCG2-mediated efflux of sulfasalazine in mice (Suneet et al. 2009) and the function of three major ABC drug transporters: Pgp, ABCG2 and MRP1 (Chearwae et al. 2004, 2006; Limtrakul et al. 2007). In the present investigation, we studied the effect of curcuminoids on purified Ha-Pgp ATPase activity, interaction and transport function.

Materials and Methods

Chemicals

ATP and ouabain were purchased from Sigma (St. Louis, MO), as were phosphatidylcholine and phosphatidic acid. All other chemicals used were of analytical grade. Turmeric rhizomes, purchased from a local market in Gulbarga, India, were dried and blended to a powder form.

Insects

Resistant pests, which have developed resistance to various insecticides (Srinivas et al. 2004) and are broadly referred to as the "resistant population," were supplied by Dr. S. S. Udikeri (Agriculture Research Station, UAS, Dharwad, India).

Extraction of Curcuminoids from Turmeric Powder

Curcuminoids were extracted following the method of Chearwae et al. (2004). The powder was extracted with 95% ethanol for 24 h. The ethanolic extract was filtered through Whatman filter paper no. 2, and ethanol was removed using a rotary evaporator. Crude curcuminoids were then purified by precipitation with petroleum ether. The precipitate was removed by filtration through Whatman filter paper no. 2 and dried at 60°C. Curcuminoid was further fractionated by silica gel 60 column chromatography, using first chloroform and then chloroform/methanol with increasing polarity to yield pure fractions of curcumins I, II and III. Fractions were collected and spotted on thin-layer chromatographic (TLC) aluminum sheets coated with silica gel 60. Fractions that showed the same pattern on TLC were pooled, and the organic solvent was removed to obtain the powder form. These curcuminoids were used in the experiments described here.

Purification of Ha-Pgp and Determination of ATPase Activity

Ha-Pgp was purified as per our earlier report (Aurade et al. 2010). ATPase activity was determined by quantitating the release of inorganic phosphate from ATP (Borgnia et al. 1996). In brief, an aliquot of enzyme was incubated in 1 ml of the ATPase assay medium containing 2.5 mM ATP, 75 mM KCl, 5 mM $MgCl_2$, 50 mM Tris–HCl, 0.5 mM EGTA, 2 mM ouabain and 3 mM sodium azide (pH 7.4) for 30 min at 37°C. The reaction was terminated by the addition to each test tube of 2 ml ice-cold stopping medium (0.2% [w/v] ammonium molybdate, 0.9% SDS, 2.3% TCA, 1.3% [w/v] sulfuric acid and freshly prepared 1% [w/v] ascorbic acid). After 30-min incubation at room temperature, the released phosphate was quantitated colorimetrically at 660 nm. To study the effect of curcuminoids, verapamil and ethylparaoxon at different concentrations were included in the reaction mixture.

Measurement of Tryptophan Quenching of Ha-Pgp

The affinity of curcuminoid binding to highly purified Ha-Pgp was determined using Trp quenching titrations as described previously (Sreeramulu et al. 2007). In brief, fluorescence intensity was measured with a Varian (les Ulis, France) fluorescence spectrophotometer. Purified Ha-Pgp was titrated in 2 mM 3([3-cholamidopropyl] dimethylammonio)-propanesulfonic acid (CHAPS) buffer and dimyristoyl-L-α-phosphatidylcholine (DMPC) with

increasing concentrations of curcumins, while quenching of Trp fluorescence emission was monitored at 330 nm following excitation at 280 nm (5 nm slit widths). ΔF_{max} and K_d values were extracted following fitting of the data to an equation describing binding to a single affinity site,

$$\Delta F/F_0 \times 100 = \frac{\Delta F/F_{max} \times (100) \times [S]}{K_d + [S]} \quad (1)$$

where $\Delta F/F_0 \times 100$ represents the percent change in fluorescence intensity relative to the initial value and after addition of curcuminoid. $\Delta F_{max}/F_0 \times 100$ is the maximum percent quenching of the fluorescence intensity that occurs upon saturation of the substrate binding site. [*S*] is the substrate concentration.

Effect of Curcuminoids on Tetramethylrosamine Transport by Ha-Pgp in Reconstituted Proteoliposomes

Purified Ha-Pgp ATPase was reconstituted into proteoliposomes and detected as described earlier (Aurade et al. 2006). Fluorescence measurements of tetramethylrosamine (TMR) transport were carried out as described previously (Sreeramulu et al. 2007). Excitation was carried out at 550 nm and fluorescence emission was monitored continuously at 575 nm (slit width, 5 nm). A 450-µl aliquot of proteoliposomes containing 10–20 µg of reconstituted Pgp was preincubated with an appropriate concentration of TMR in the transport buffer at room temperature. The sample was transferred to a quartz cuvette and allowed to equilibrate for about 300 s to stabilize the fluorescence intensity. The addition of a 25-µl aliquot of buffer containing ATP (final concentration 1 mM) and an ATP-regenerating system (30 µg/ml creatine kinase and 3.5 mM creatine phosphate) followed by mixing for 5–10 s initiated the transport of TMR. Fluorescence intensity data were collected for a further 150–200 s. Fluorescence intensity vs. time was normalized to the intensity measured immediately before addition of ATP, which was taken as 100%. The relative rate of TMR transport was estimated from the slope of the fluorescence traces for the first 20 s after the addition of ATP. Different concentrations of curcuminoids were added at various time points during the transport process, either before the addition of ATP or after the establishment of a new steady-state fluorescence value. In the latter case, fluorescence intensity data were collected for a further 150–200 s.

Results

Effect of Curcuminoids on Ha-Pgp ATPase Activity

Curcuminoids were purified by silica gel 60 column chromatography to yield pure forms of curcumins I, II and III. Further, their purity was assessed by TLC (Fig. 1). The curcumin mixture and individual curcumins (I, II and III) stimulated Ha-Pgp ATPase activity at lower concentrations (0.5–5 µM) but inhibited the level of basal activity at higher concentrations (Fig. 2). The curcumin mixture inhibited 90% Ha-Pgp ATPase activity compared with curcumins I, II and III, which inhibited 70–80% at 100 µM concentration. Purified curcuminoids inhibited verapamil- and ethylparaoxon-stimulated ATPase activity in a concentration-dependent manner. The curcumin mixture inhibited verapamil (30 µM)–stimulated ATPase activity by 80–90% compared with individual curcumins (I, II and III), which inhibited the activity by 70, 50 and 40%, respectively (Fig. 3a). The curcumin mixture inhibited ethylparaoxon (50 µM)–stimulated ATPase activity by 100% compared with individual curcumins I, II and III, which inhibited the activity by 80, 70 and 60%, respectively (Fig. 3b). Verapamil (Dong et al. 1996) and ethylparaoxon (Aurade et al. 2010) were known to stimulate Pgp-ATPase activity at lower concentrations.

Effect of Curcuminoids and ATP on Intrinsic Ha-Pgp Trp Fluorescence

Quenching of Ha-Pgp tryptophan fluorescence emission was monitored at 330 nm and excitation at 280 nm. The addition of ATP to Pgp resulted in a large quenching of fluorescence (Fig. 4) and suggests interaction of ATP with Pgp, as expected (Sharom et al. 1998). Ha-Pgp, in the presence of all curcuminoids (curcumins I, II, III and

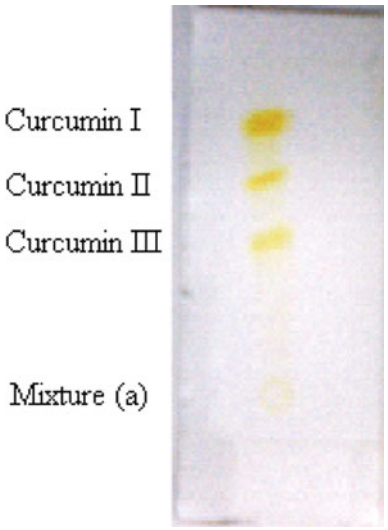

Fig. 1 Determination of curcuminoids by thin-layer chromatography (TLC). Ethanolic extracts were spotted on silica gel Merck TLC in the solvent system of chloroform/ethanol/acetic acid (90:5:1). (a), the origin of the curcumin mixture; I, II and III, positions of curcumins I, II and III

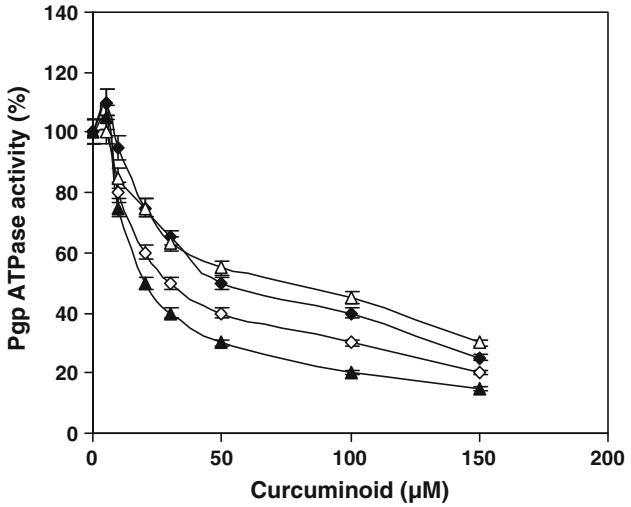

Fig. 2 Effect of curcuminoids on Ha-Pgp ATPase activity. *Open diamond* curcumin I, *filled diamond* curcumin II, *open triangle* curcumin III, *filled triangle*, curcumin mixture. Purified Ha-Pgp was incubated with increasing concentrations of curcumins I, II and III and the curcumin mixture (0–150 μM) at 37°C for a few minutes; the reaction was initiated by addition of 2.5 mM ATP. Data represent mean ± SD of three independent experiments

curcumin mixture), also showed large quenching (Fig. 4). A small shift of 1–4 nm was observed toward the left on the emission maximum with curcuminods (Fig. 4). The results suggest that curcuminoids are modulators with bifunctional interactions at vicinal ATP-binding sites and steroid–interacting regions. These are expected to be in close proximity to the ATP-binding site within a cytosolic domain of Pgp (Conseil et al. 1998; Boumendjel et al. 2002).

Measurement of Interaction of Curcuminoids with Ha-Pgp

Trp quenching experiments were carried out using the curcumin mixture and curcumins I, II and III; and the K_d values for binding were estimated by fitting the fluorescence quenching data to an equation describing a single binding site (Fig. 5a, b). These compounds displayed substantial levels of Trp quenching. Fitting of the experimental data showed that all four compounds interacted with Pgp with relatively high affinity, with K_d values for binding in the 4–10 μM range in the absence of ATP and in the 2–5 μM range in the presence of ATP (Fig. 5a, b).

Effect of Curcuminoids on TMR Transport by Pgp in Proteoliposomes

In previous studies, TMR was chosen as a substrate for measuring Pgp-mediated transport into reconstituted proteoliposomes in vitro (Lu et al. 2001). The addition of

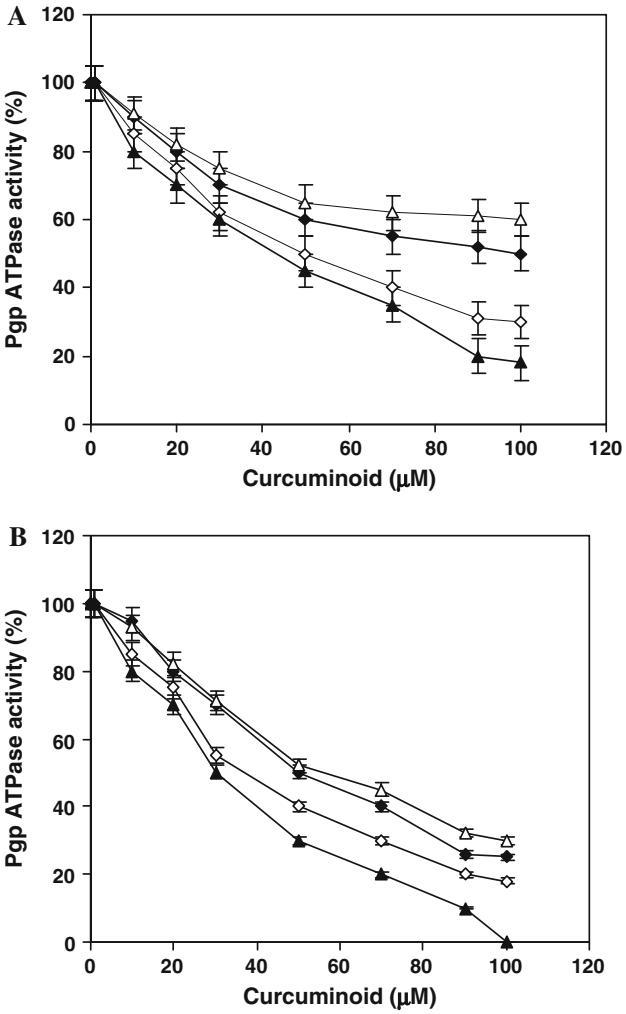

Fig. 3 Inhibitory effect of curcuminoids on verapamil and paraoxon-stimulated Ha-Pgp ATPase activity. **a** Verapamil-stimulated: *open diamond* curcumin I, *filled diamond* curcumin II, *open triangle* curcumin III, *filled triangle* curcumin mixture. **b** Ethylparaoxon-stimulated: *open diamond* curcumin I, *filled diamond* curcumin II, *open triangle* curcumin III, *filled triangle* curcumin mixture. Purified Ha-Pgp was incubated with increasing concentrations of curcumin mixture (0–100 μM) in the presence of 30 μM verapamil in ATPase assay buffer. Similarly, ethylparaoxon-stimulated, purified Ha-Pgp was incubated with increasing concentrations of curcumin mixture (0–100 μM) in the presence of 50 μM ethylparaoxon. The data represent mean ± SD of three independent experiments

1 mM ATP and an ATP–regenerating system to reconstituted DMPC proteoliposomes containing Ha-Pgp in the presence of 10 μM TMR led to a rapid drop in TMR fluorescence until a new lower steady–state level was established (Fig. 6). The decrease in fluorescence intensity arises from transport of TMR into the proteoliposomes, where its fluorescence is lower, likely as a result of self-quenching as it accumulates in the lumen. No decrease in TMR fluorescence was observed in the presence of non-hydrolyzable ATP analogs; heat denaturation of Pgp

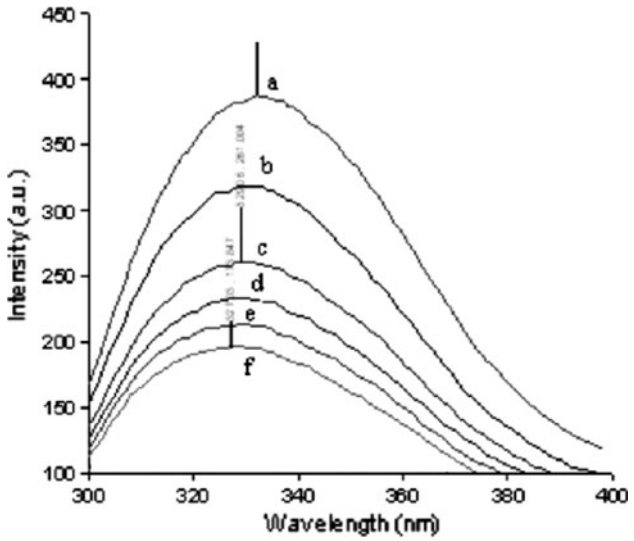

Fig. 4 Fluorescence emission spectra of Ha-Pgp Trp residues in the absence (*a*) and presence of 1 mM of ATP (*b*), 10 μM curcumin I (*c*), 10 μM curcumin II (*d*), 10 μM curcumin III (*e*) and 10 μM curcumin mixture (*f*)

abolished the change in TMR fluorescence (data not shown). The process of TMR transport into proteoliposomes is dependent on ATP hydrolysis and requires functionally active Pgp. We examined the effect of vanadate and curcumin on TMR transport by Ha-Pgp proteoliposomes; curcumin at concentrations that block transport of TMR into the proteoliposome interior could be predicted to prevent the decrease of rapid fluorescence when added prior to ATP (Fig. 6a, b).

Discussion

Among the several enzymes reported, Ha-Pgp was one of those responsible for insecticide resistance in *H. armigera* (Srinivas et al. 2004). Since *H. armigera* acquired resistance to almost all synthetic insecticides, further application of any other insecticides will not only increase the fold of resistance but also make it extremely difficult to control them. There were no reports on the inhibition of Pgp ATPase activity of *H. armigera* by any bioactive compounds. Therefore, the search for novel and more potent Ha-Pgp modulators is of major importance. In this study, we focused on curcuminoids, which are polyphenolic pigments found in the spice turmeric. The curcumin mixture could reverse multidrug resistance in KB-VI cells by inhibiting function (Anuchapreeda et al. 2002) and inhibited human P-gp (ABCB1).

Curcumins I, II and III stimulated human Pgp ATPase activity below 1 μM and inhibited ATPase activity up to 90% at 30 μM (Chearwae et al. 2004). Similarly, they also

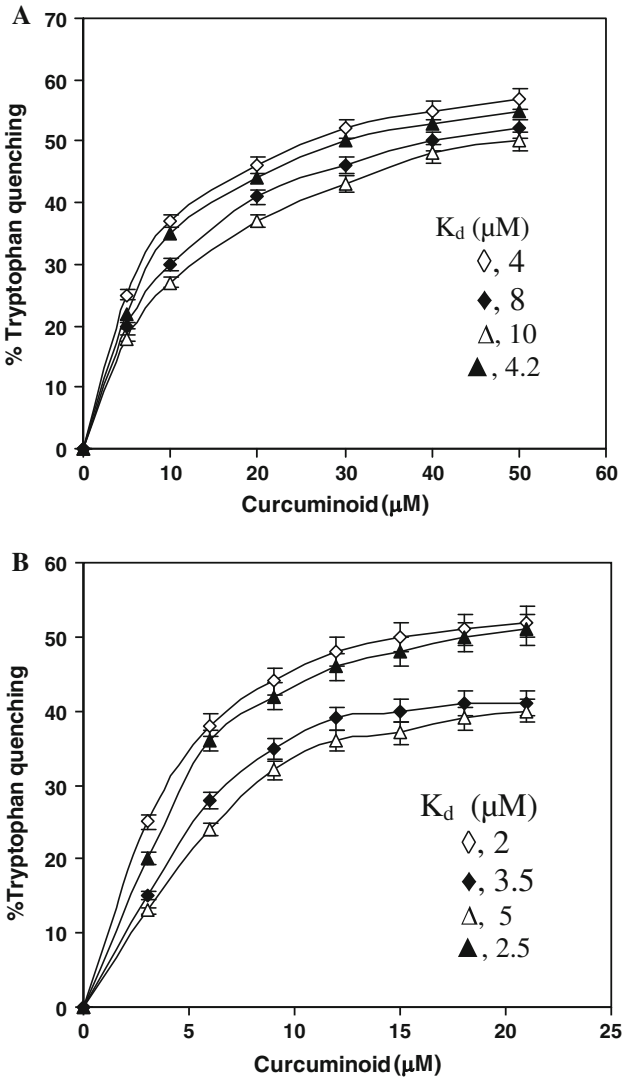

Fig. 5 Binding of curcuminoid to purified Ha-Pgp as assessed by tryptophan fluorescence quenching (**a**) in the absence and (**b**) in the presence of ATP. *Open diamond* curcumin I, *filled diamond* curcumin II, *open triangle* curcumin III, *filled triangle* curcumin mixture. Increasing concentrations of curcumin were added at 22°C to 50 μg/ml of Pgp in Tris–HCl buffer, pH 7.4. *Continuous line* represents the best computer-generated fit of the data points (shown by the *symbols*) to an equation describing the interaction with a single type of binding site. The data represent mean ± SD of two independent experiments

stimulated Ha-Pgp ATPase activity below 5 μM and inhibited Ha-Pgp ATPase activity up to 80–90% at 100 μM concentration. The curcuminoids inhibited ATPase activity in a concentration-dependent manner. They also inhibited verapamil- and ethylparaoxon-stimulated Ha-Pgp ATPase activity at higher concentrations, suggesting that the binding site for the three curcuminoids may overlap that of verapamil and ethylparaoxon (Fig. 3a, b). Thus, the binding of curcuminoids may change the conformation of Pgp and affect the binding of other drug substrates such as verapamil.

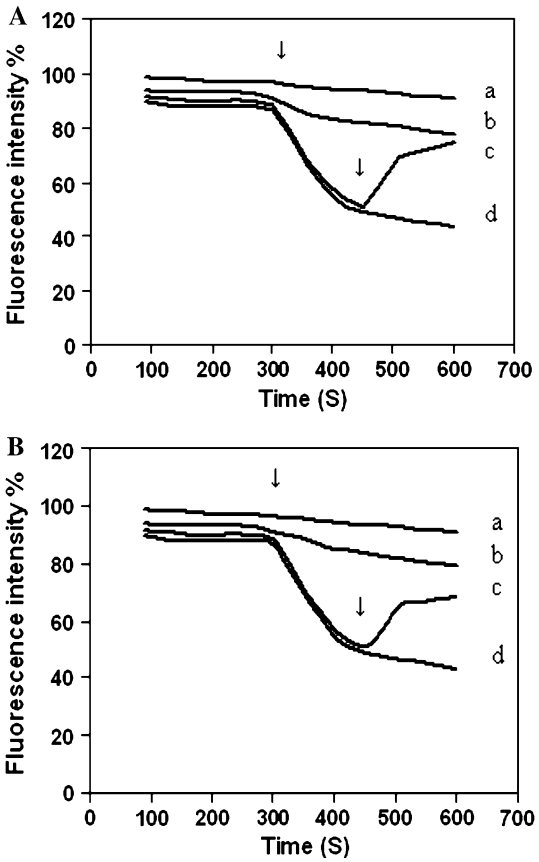

Fig. 6 Effect of vanadate (**a**) and curcumin (**b**) on TMR transport in reconstituted proteoliposomes containing Ha-Pgp. Other details are mentioned in Materials and Methods. An aliquot of buffer was added (indicated by an *arrow*) containing either vanadate (**a**, concentration 10 μM) or curcumin mixture (**b**, concentration 50 μM), and after mixing, further fluorescence intensity data were collected. **a** Trace *a* (TMR + vanadate + ATP), 50 μM vanadate was added to the proteoliposomes before the addition of 1 mM ATP; trace *b* (TMR + vanadate + ATP), 10 μM vanadate was added to the proteoliposomes before the addition of ATP; trace *c* (TMR + ATP + vanadate), ATP was added to the proteoliposomes to initiate TMR transport (shown by an *arrow*), a lower fluorescence intensity was reached after 150 s as a result of inward pumping of TMR, and 50 μM vanadate was then added (shown by an *arrow*), which inhibits Pgp, collapsing the TMR gradient; trace *d*, control (TMR + ATP). **b** Similarly for curcumin mixture, trace *a* (TMR + curcumin + ATP); trace *b* (TMR + curcumin + ATP); trace *c* (TMR + ATP + curcumin); trace *d*, control (TMR + ATP)

The Ha-Pgp tryptophan fluorescence quenching measurement data indicate that curcuminoids may bind with ATP-binding sites and steroid-interacting regions (Fig. 4). Flavonoids may induce their binding affinity toward nucleotide binding domain 2 of Pgp through their ability to mimic the adenine moiety of ATP. Therefore, a hydrophobic moiety of polyphenols may be important for interaction at the steroid-interacting hydrophobic sequences of Pgp (Conseil et al. 1998; Boumendjel et al. 2002). Hydroxyl groups in polyphenols may be important in polar interactions with Pgp, possibly at the ATP-binding site. The tryptophan fluorescence measurement data indicate that all three curcuminoids bind to Ha-Pgp with high affinity (Fig. 5). The structure of curcumin I looks more suitable for binding to the drug-binding site of Ha-Pgp than those of curcumins II and III as curcumin I has a balance of two hydroxyl and methoxyl groups on each side and the presence of two methoxyl groups in the curcumin I molecule might help its inhibitory activity on Pgp function (Chearwae et al. 2004). Since curcumin I is a major component (> 70%) of the curcumin mixture (Chearwae et al. 2004) and its efficacy as a modulator is similar to that of the curcumin mixture, the presence of both curcumins II and III in the mixture should not diminish the effect of curcumin I. The transport of a drug substrate by Pgp is coupled to ATP hydrolysis; there is evidence for stimulation of ATPase activity of Pgp by drug substrates or modulators from diverse systems (Sauna and Ambudkar 2001). The low K_d values in the presence of ATP reported here would further support that the interaction of curcuminoids with Ha-Pgp is very high. Comparing the K_d values obtained in the absence and presence of ATP, it is possible to conclude that the degree of affinity in the presence of ATP depends on the compound under investigation.

The curcumin mixture inhibited TMR transport in proteoliposomes containing Ha-Pgp. Curcumin I is the most active form for increasing the intracellular levels and cytotoxicity of vinblastine in KB–V1 cells by modulating Pgp function. The present study establishes that the fluorescent dye TMR is a high-affinity substrate for Ha-Pgp and shows that it can be used to carry out real-time transport measurements in proteoliposomes containing Ha-Pgp. The high sensitivity of this technique is suitable for reconstituted systems; it has several advantages over other methods used previously to examine transport in proteoliposomes (Lu et al. 2001). In the present study, we demonstrate that Ha-Pgp generates a substrate concentration gradient by inward pumping of the fluorescent substrate TMR into reconstituted proteoliposomes. The addition of 100 μM curcumin mixture, which inhibits Ha-Pgp ATPase activity, blocks Ha-Pgp-mediated transport of TMR into the interior of proteoliposomes. The addition of vanadate or curcuminoid (Fig. 6a, b) to proteoliposomes following the establishment of the new steady state resulted in rapid restoration of TMR florescence, indicating collapse of the TMR gradient. The addition of MDR modulators verapamil and cyclosporin A also led to a similar increase in fluorescence (Lu et al. 2001). The ATPase activity of the inward-facing Pgp molecule is inhibited, and the ADP.Vi.Mg^{2+} complex is trapped at one active site after a single catalytic turnover (Urbatsch et al. 1995). The potency of curcuminoids is comparable to that of vanadate, which is a known inhibitor of Pgp-mediated transport with

high affinity. Thus, the presence of vanadate or curcuminoids outside the proteoliposomes blocks inward pumping by Ha-Pgp, leading to collapse of the previously established gradient.

Curcuminoids have been reported to be potent inhibitors of Pgp- or MRP1- mediated transport (Chearwae et al. 2004, 2006; Hong et al. 2003; Wortelboer et al. 2003). However, this is the first report, to our knowledge, where the purified curcumin mixture and forms I, II and III have been used to assess their effect on the function of Ha-Pgp. Thus, these biochemical results demonstrate that curcuminoids interact directly with Ha-Pgp and possibly bind to the same sites as the ATP or other agents such as verapamil. In conclusion, our results show that all of the forms of curcuminoids can inhibit Ha-Pgp function and have beneficial effects in the management of insecticide-resistant pests.

Conclusion

Purified curcuminoids (I, II and III) were found to be potent inhibitors of Ha-Pgp ATPase. They interact with Ha-Pgp with relatively high affinity, with K_d values of 2–10 μM. Curcuminoids also inhibit TMR transport in reconstituted proteoliposomes. Hence, these bioactive compounds can be used in the future in place of synthetic chemicals to overcome resistance in this pest.

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