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Cooperativity between verapamil and ATP bound to the efflux transporter P-glycoprotein

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ABSTRACT

The P-glycoprotein (Pgp) transporter plays a central role in drug disposition by effluxing a chemically diverse range of drugs from cells through conformational changes and ATP hydrolysis. A number of drugs are known to activate ATP hydrolysis of Pgp, but coupling between ATP and drug binding is not well understood. The cardiovascular drug verapamil is one of the most widely studied Pgp substrates and therefore, represents an ideal drug to investigate the drug-induced ATPase activation of Pgp. As previously noted, verapamil-induced Pgp-mediated ATP hydrolysis kinetics was biphasic at saturating ATP concentrations. However, at subsaturating ATP concentrations, verapamil-induced ATPase activation kinetics became monophasic. To further understand this switch in kinetic behavior, the Pgp-coupled ATPase activity kinetics was checked with a panel of verapamil and ATP concentrations and fit with the substrate inhibition equation and the kinetic fitting software COPASI. The fits suggested that cooperativity between ATP and verapamil switched between low and high verapamil concentration. Fluorescence spectroscopy of Pgp revealed that cooperativity between verapamil and a non-hydrolyzable ATP analog leads to distinct global conformational changes of Pgp. NMR of Pgp reconstituted in liposomes showed that cooperativity between verapamil and the non-hydrolyzable ATP analog modulate each other's interactions. This information was used to produce a conformationally-gated model of drug-induced activation of Pgp-mediated ATP hydrolysis.

1. Introduction

P-glycoprotein (Pgp) is an ATP hydrolysis-driven efflux transporter that is part of the ATP-binding cassette (ABC) superfamily of proteins [1,2]. The transporter effluxes a chemically and structurally diverse range of molecules, including anticancer drugs, neurotherapeutics and cardiovascular drugs, from the cytosol to the extracellular space across cell membranes [1,2]. The transporter is expressed at relatively high concentrations in the brain, intestines, liver, placenta, and the kidneys [3,4]. Pgp expression level is also influenced by genetic polymorphisms and disease [5,6]. The transporter functions to protect tissues from chemical toxicity, but also leads to drug resistance and can significantly affect drug disposition [1,2]. For example, the transporter protects the brain from chemical insults by effluxing drugs across the blood–brain barrier (BBB) [7], but also makes cancerous tumors overexpressing the protein resistant to anticancer drugs [8]. As a result, there has been keen interest in unraveling the molecular and structural basis of transport with Pgp. This knowledge is critical for the development of novel transport inhibitors, drugs with desirable transport properties and improving predictions of *in vivo* drug disposition from *in vitro* measurements.

Most of our structural understanding of the transporter comes from X-ray crystallography of mouse Pgp (Abcb1a), *Caenorhabditis* (*C.*) *elegans* Pgp and bacterial transporters [9–12]. The X-ray crystal structure of mouse Pgp revealed a 170 kD pseudosymmetric monomer, consisting of two nucleotide-binding domains (NBDs) and 12

Abbreviations: AMPPNP, adenosine 5′-(β,γ-imido)triphosphate; COPASI, complex pathway simulator; DDM, *n*-dodecyl-β-*D*-maltoside; $F_{0,H}$, initial fluorescence intensity at the high concentration phase; $F_{0,L}$, initial fluorescence intensity at the low concentration phase; K_A, association constant; K_D, dissociation constant; K_H, equilibrium constant at high concentration; K_L, equilibrium constant at low concentration; K_{SV}, Stern-Volmer quenching constant; *L*, ligand; NTA, nickel-nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; Pgp, P-glycoprotein; *Pi*, inorganic phosphate; RF, radio frequency; SDS, sodium dodecyl-sulfate; *Q*, quenching ligand; STD, saturation transfer difference; STDD, saturation transfer double difference.

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transmembrane (TM) helices [9]. The bacterial transporter X-ray crystal structures of MsbA and SAV1866 have only 6 TM helices, but as dimers these proteins resemble the 3-dimensional fold of mammalian Pgps [10,11]. The bacterial transporter X-ray crystal structures have also been found in different conformations with nucleotide cofactors suggesting that conformational changes play a role in transport [10,11,13]. Since both bacterial and mouse Pgp have conserved motifs within the NBDs, including Walker A, Walker B and ABC signature motifs e.g. [14], they are considered to have similar transport mechanisms. From these bacterial transporter structures, a conformationally gated transport model was proposed [10,11].

Despite the availability of X-ray crystal structures, our understanding of the coupling between drug binding, ATP hydrolysis and transport remains limited. Cross-linking studies of Pgp in human embryonic kidney (HEK) 293 cell membranes suggest that drug-induced conformational changes can occur with the NBDs or the transmembrane region [15,16]. A study on human Pgp in nanodiscs with antibodies showed that there are ligand and cofactor-dependent conformational changes [17]. A fluorescence study with mouse Pgp found differences in fluorescence resonance energy transfer (FRET) with drugs, nucleotide cofactors and their analogs suggesting conformational changes [18].

In addition to being substrates for the transporter, a number of drugs are known to activate ATP hydrolysis of Pgp, but little is known of the molecular mechanism or its relationship to transport. One of the most studied drugs is the cardiovascular drug verapamil (Fig. 1A), which is used to treat hypertension, chest pain and arrhythmia [19–22], and can function as both a substrate and an inhibitor of the transporter [23]. The drug is known to activate Pgp-coupled ATP hydrolysis from a number of *in vitro* studies e.g. [24,25]. The kinetics of verapamil-induced Pgp-coupled ATP hydrolysis is biphasic [24–27] suggesting at least two verapamil binding sites. Biphasic drug-induced ATPase activation kinetics has been observed with a chemically diverse range of substrates from amitriptyline to vinblastine e.g. [24,28,29] implying a common mechanism between these types of substrates and Pgp. Despite the large number of studies, the molecular basis for drug-induced ATPase activation of Pgp and the underlying interactions between drugs, ATP and Pgp are not well understood.

In the proposed studies, the interactions of verapamil and ATP were investigated with Pgp reconstituted into liposomes. To investigate the coupling between verapamil and ATP, verapamil-induced activation of ATPase activity was examined with a range of verapamil and ATP concentrations. Acrylamide quenching of intrinsic tryptophan fluorescence spectroscopy was used to investigate drug and nucleotide-induced conformational changes of Pgp. The interactions between verapamil and a non-hydrolyzable ATP analog were investigated by the saturation transfer double difference (STDD) NMR technique. These results were used to build a model of verapamil-induced ATPase activation of verapamil efflux by the transporter. Since similar biphasic drug-induced ATPase activation is observed for several Pgp substrates, this mechanism will likely be generalizable e.g. [24,28,29].

2. Materials and methods

2.1. Materials

Adenosine 5′-(β,γ-imido)triphosphate lithium salt (AMPPNP) was purchased from Sigma Aldrich (Milwaukee, WI) and verapamil hydrochloride was purchased from Fagron (St. Paul, MN). The detergent *n*-dodecyl-*β*-*D*-maltoside (DDM), which is used in protein purification, was purchased from EMD Millipore Corporation (San Diego, CA). *Escherichia coli* (*E. coli*) total lipid extract powder was purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and cholesterol was purchased from Amresco (Solon, OH) for liposome preparations. Disodium ATP (Na_2ATP) was purchased from Amresco (Solon, OH) and sodium orthovanadate (Na_3VO_4) was purchased from Enzo Life Sciences (Farmingdale, NY) for the

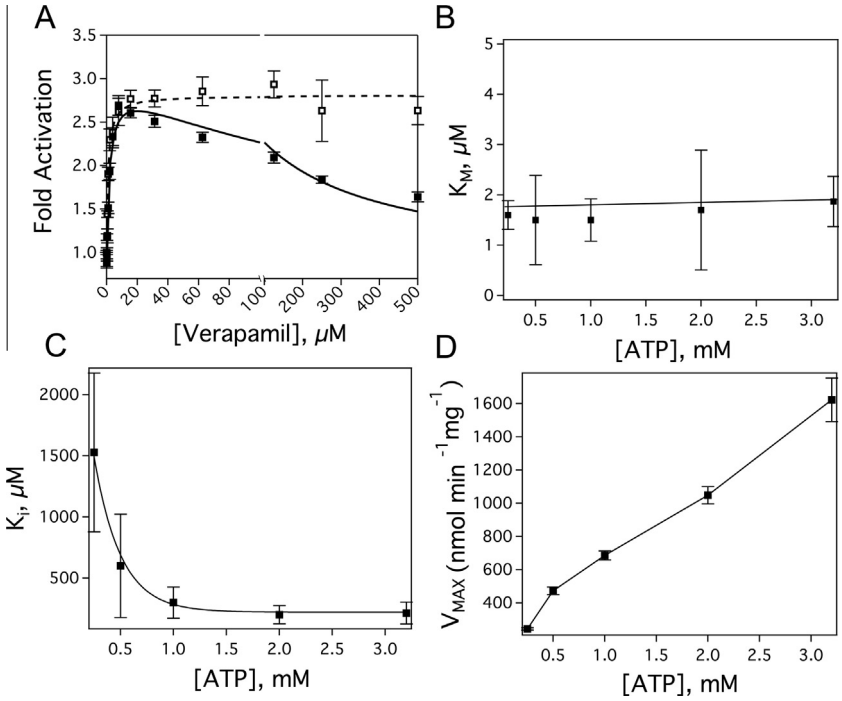

Fig. 1. The effect of ATP on verapamil-induced ATPase activation of Pgp. (A) The Pgp-coupled ATPase activity as a function of verapamil concentration in the presence of 3.2 mM (closed squares) and 0.25 mM ATP (open squares). The kinetic fits are shown as a dashed and solid line and were fit to Eqs. (1) and (2), respectively. (B) The Michaelis-Menten constant (K_m), (C) the inhibition constant (K_i) and (D) the V_{MAX} for verapamil-induced Pgp ATPase activity as a function of different ATP concentrations. Error bars represent the standard deviation and the points represent the average of at least three independent experiments.