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Evidence for Multidrug Resistance-1 P-Glycoprotein-dependent Regulation of Cellular ATP Permeability

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Abstract. The mechanisms responsible for regulating epithelial ATP permeability and purinergic signaling are not well defined. Based on the observations that members of the ATP-binding cassette (ABC)[1] family of proteins may contribute to ATP release, the purpose of these studies was to assess whether multidrug resistance-1 (MDR1) proteins are involved in ATP release from HTC hepatoma cells. Using a bioluminescence assay to detect extracellular ATP, increases in cell volume increased ATP release ~3-fold. The MDR1 inhibitors cyclosporine A (10 μM) and verapramil (10 μM) inhibited ATP release by 69% and 62%, respectively ($p < 0.001$). Similarly, in whole-cell patch-clamp recordings, intracellular dialysis with C219 antibodies to inhibit MDR1 decreased ATP-dependent volume-sensitive Cl^- current density from -33.1 ± 12.5 pA/pF to -2.0 ± 0.3 pA/pF (−80 mV, $p \leq 0.02$). In contrast, overexpression of MDR1 in NIH 3T3 cells increased ATP release rates. Inhibition of ATP release by Gd^{3+} had no effect on transport of the MDR1 substrate rhodamine-123; and alteration of MDR1-substrate selectivity by mutation of G185 to V185 had no effect on ATP release. Since the effects of P-glycoproteins on ATP release can be dissociated from P-glycoprotein substrate transport, MDR1 is not likely to function as an ATP channel, but instead serves as a potent regulator of other cellular ATP transport pathways.

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[1]*Abbreviations:* ABC, ATP-binding cassette proteins; Pgp, P-glycoprotein; spgp, sister of P-glycoprotein; MDR1, multidrug resistance-1 P-glycoprotein; CFTR, cystic fibrosis transmembrane conductance regulator; NGFR, nerve growth factor receptor

Key words: ABC protein — Cell volume — Liver — Purinergic receptor — Cl^- channel

Introduction

In many epithelial cells, ATP is released into the extracellular milieu where it functions as a signaling molecule regulating a broad range of cellular functions through stimulation of purinergic receptors (Harden, Boyer & Nicholas, 1997). Despite great interest in the mechanisms that regulate cellular ATP permeability, the molecular basis for epithelial ATP transport is presently unknown.

ATP-binding cassette (ABC) transporters, including the cystic fibrosis transmembrane conductance regulator (CFTR) and multidrug resistance (MDR) P-glycoproteins (Pgp), have been proposed to contribute to cellular ATP release in some cell model systems (Roman & Fitz, 1999). The role of CFTR in this respect has been controversial. While initial reports indicated that ATP permeability and ATP-selective currents increased with native and heterologous expression of CFTR (Reisin et al., 1994; Pasyk & Foskett, 1997; Schwiebert et al., 1995), the ability of CFTR to conduct ATP has been questioned by others (Grygorczyk & Hanrahan, 1997; Reddy et al., 1996). More recently, by expressing CFTR in MDCK cells, Sugita and coworkers demonstrated a close association between plasma membrane CFTR expression and ATP currents, and proposed that CFTR Cl^- channel pores and ATP channel pores are distinct but commonly gated (Sugita, Yue & Foskett, 1998).

It is clear that CFTR is not required for ATP release since primary human hepatocytes and other cells with no detectable expression of CFTR are capable of regulating

ATP permeability in response to changes in phophoinosityl-3 kinase and cell volume (Feranchak et al., 1998; Roman & Fitz, 1999). Many of these tissue express P-glycoproteins encoded by MDR genes. Although the potential role of P-glycoproteins as modulators of cellular ATP permeability has been less well-studied, there is evidence that P-glycoproteins may conduct ATP (Abraham et al., 1993). In HTC hepatoma cells, volume-sensitive Cl^- currents are ATP-dependent and are attenuated by inhibition of P-glycoproteins with verapamil or cyclosporin A (Wang et al., 1996; Roman et al., 1997). Moreover, upregulation of multiple P-glycoproteins in a related HTC-R hepatoma cell line results in enhanced ATP permeability and cell volume recovery (Roman et al., 1997). Based on these observations, the purpose of these studies was to address the potential role for MDR1 P-glycoproteins as regulators of cellular ATP permeability.

Materials and Methods

Reagents

Verapamil, cyclosporin A, gadolinium chloride, lanthanum chloride, and rhodamine 123 were obtained from Sigma (St. Louis, MO). Luciferase-luciferin was purchased as a component of an ATP-assay kit from Calbiochem (San Diego, CA).

Antibodies

Monoclonal antibodies utilized to inhibit P-glycoprotein function included: i) C219 antibodies, which bind to cytoplasmic epitopes of P-glycoprotein near the nucleotide-binding domains and inhibit ATP hydrolysis (Signet) (Vanoye, Altenberg & Reuss, 1997), and ii) UIC2 antibodies, which recognize a conformational extracellular epitope of human MDR1 P-glycoprotein and block substrate transport, were provided by Eugene Mechetner, Oncotech, Inc., Irvine, CA (Mechetner & Roninson, 1992; Zhou, Gottesman & Pastan, 1999). Polyclonal rabbit antibodies to β-galactosidase (5 Prime → 3 Prime, Inc.®) were used as controls for C219 (patch-clamp studies). For bioluminescence experiments using UIC2, monoclonal UPC10 antibodies (also IgG2a, Sigma) were added as controls. All antibodies were dialyzed thrice against growth medium without serum to remove azide.

Cell Models

Studies in isolated cells were performed using rat HTC hepatoma cells as described previously (Wang et al., 1996). Models of P-glycoprotein overexpression included bile-acid resistant rat HTC-R cells, which overexpress multiple endogenous and novel mdr proteins (Brown et al., 1995; Roman et al., 1997), and human NIH 3T3 fibroblasts transfected with wild-type human MDR1. For MDR1 expression, a truncated version of the Nerve Growth Factor Receptor (NGFR) was cloned into pLNCX Vector (Clontech, Palo Alto, CA; named pLNGFR), and human MDR1 cDNA was subsequently cloned into the pLNGFR vector. MDR1 and NGFR expression are driven by CMV and retroviral (LTR) promoters, respectively. These clones were used to make mock (NGFR) and MDR1-expressing NIH 3T3 cells (NGFR/MDR1). The rat canalicular bile salt transporter sister of P-glycoprotein (spgp) was cloned into the pCR3 vector (Invitrogen, Carlsbad, CA) with expression driven by the CMV promoter. NIH 3T3 and human embryonic kidney (HEK) 293 cells were transfected with MDR1 and sgpg, respectively, using the Lipofectamin reagent (Gibco-BRL,). For other studies, NIH 3T3 cells stably expressing mutant MDR1 P-glycoprotein (V185), were compared to cells expressing wild-type MDR1 (G185). These cell lines were a generous gift from Suresh Ambudkar (National Institutes of Health, Bethesda, MD). NIH 3T3 and HEK 293 cells were grown in MEM/H21 with 4 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1.0 mM sodium pyruvate, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal bovine serum. For MDR1-expressing cells, 1 μg/ml colchicine was added to media to maintain high mdr levels. HTC and HTC-R cells were maintained as previously described (Roman et al., 1997).

Solutions

The standard extracellular NaCl solution used for most studies contained (in mM): 140 NaCl, 4 KCl, 1 KH_2PO_4, 2 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 10 HEPES/NaOH (pH 7.40) with a total of Cl^- of 150 mM. Solution osmolarity (vapor-pressure osmometer, Model 5500, Wescor,) was ~295 mosmol. Cells were exposed to hypotonic stress by lowering buffer NaCl concentration (patch clamp) or adding water to extracellular media (luciferase-luciferin assay). Extracellular hypotonicity (10–60% reductions in osmolarity) did not affect cell viability over 1 hr (propidium iodide staining, *data not shown*).

Western Blot Analysis

Cells grown to confluence were harvested by scraping, rinsed once in PBS buffer, and sonicated. Nuclei and unlysed cells were removed by centrifugation (400 × g, 10 min at 4°C), an aliquot of the resulting supernatant was stored for total-proteins analysis and the remainder was centrifuged (100,000 × g, 30 min at 4°C). The resulting crude membrane pellet was resuspended in buffer (50 mM Tris, 50 mM Mannitol, pH 7.0). All solutions were supplemented with the proteinase inhibitors leupeptin (4 μM), pepstatin (2 μM), EGTA (2 mM), and phenylmethylsulfonyl fluoride (0.5 mM) (Sigma). Total proteins (40 μg) isolated from resistant (HTC-R), nonresistant (HTC), MDR1/NIH 3T3, and parental NIH 3T3 cells were each fractionated in duplicate by electrophoresis on 7.5% SDS-PAGE. The first gel was used for Coomassie Blue staining, and the second gel was transferred to nitrocellulose membrane by electroblotting (Towbin, Staehelin & Gordon, 1989). The nitrocellulose membrane was incubated with a 1:500 dilution of mouse monoclonal antibody C219 (Signet, Dedham, MA) against the human P-glycoprotein (Georges et al., 1990). Immune complexes were detected using anti-mouse antibodies coupled to alkaline phosphatase.

Bioluminescence ATP Detection Assay

ATP in extracellular medium was detected by methods recently described (Taylor et al., 1998; Roman et al., 1999). Cells were grown to confluence in 35-mm dishes. Prior to study, cells were washed twice with PBS and 600 μl of serum-free Optimem-1 (Gibco-BRL) containing firefly luciferase-luciferin (lyophilized reagent, Calbiochem) was added to detect cellular ATP release. The absence of serum was essential as it inhibits the luciferase-luciferin reaction and allows fixation of complement by UIC2 antibodies. Cell-containing dishes were placed on a platform and inserted directly into a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). In complete darkness, cumulative bioluminescence over 15-sec intervals was quantified as arbitrary light

units (ALU). Hypotonic stress was induced by adding water to dilute medium; identical volumes of isotonic medium were added in control studies to dissociate volume- from mechano-sensitive ATP release. All solutions added to cells (medium, water, reagents) contained the same concentration of luciferase-luciferin, so that the reagent would not be diluted. None of the reagents that were added to media affected luminescence detected in the absence of cells using ATP standards.

Protein Measurement

To assure that relative differences in detected bioluminescence were not secondary to variations in cell content, at the end of each experiment cells were lysed to measure total protein/dish. Cells were solubilized from 35-mm dishes with 1.0 ml of 0.5% sodium dodecyl sulfate and assayed for their protein concentration using a bicinchoninic acid assay (Pierce Biochemicals, Rockford, IL). Albumin was used to standardize the values.

Measurement of Cl^- Currents

Membrane Cl^- currents were measured and analyzed using whole-cell patch-clamp techniques as previously described (Wang et al., 1996; Roman et al., 1997). Isolated HTC cells plated on coverslips were studied after ~24 hours in a chamber perfused with the extracellular buffer described above (chamber volume ~400 μl, flow 4–5 ml/min). For measurement of Cl^- currents, the intracellular (pipette) solution contained (in mM): 130 KCl, 10 NaCl, 2 $MgCl_2$, 10 HEPES/KOH, 1 ATP, 0.5 $CaCl_2$ and 1 EGTA (pH 7.3), corresponding to a free $[Ca^{2+}]$ of ~100 nM. Inward currents at −80 mV were measured to quantitate changes in Cl^- current (pA) and current density (pA/pF). Only cells with a whole-cell series access resistance ≤10 mΩ were used to assure rapid equilibration of the pipette solution with the cell interior.

Rhodamine-123 Efflux

Assay of MDR1 P-glycoprotein function was assessed by measuring the efflux rate of Rhodamine-123 (R123, Molecular Probes). MDR1-expressing NIH 3T3 cells were preincubated for 1 hour at 37°C in DME H21 medium containing 10% newborn bovine serum and R123 (10 μM). Subsequently, cells were washed six times with ice-cold PBS and R123 efflux was initiated by adding 1 ml/well prewarmed DME H21 medium containing 10% newborn bovine serum. This efflux medium was collected and replaced by fresh efflux medium at 1, 5, 10, 30 and 60 minutes. Fluorescence was measured using the Fluorolog 2 spectrofluorometer (Spex).

Statistics

Results are presented as the means ± SEM, with n representing the number of cells for patch-clamp studies and the number of repetitions for other experiments. All experiments were repeated on two or more study days. Paired, unpaired Student's t-test, and unpaired ANOVA with Bonferroni ad-hoc test were used to assess statistical significance as indicated. p Values of $p < 0.05$ were considered to be significant. *NS* designation indicates not significantly different.

Results

P-glycoprotein Inhibitors Attenuate ATP Permeability

Like rat hepatocytes in vivo, HTC hepatoma cells in culture express mdr1b P-glycoproteins. In previous studies, exposure of HTC cells to the putative P-glycoprotein inhibitors verapamil and cyclosporin A decreased activation of membrane Cl^- currents and cell volume recovery during hypotonic exposure (Roman et al., 1997). Since both of these responses require cellular ATP release and autocrine stimulation of P2 receptors (Wang et al., 1996), the luciferase-luciferin ATP detection assay was utilized to directly measure basal and volume-sensitive ATP release in these cells, and to determine whether pharmacologic inhibitors of P-glycoproteins attenuate cellular ATP permeability. As shown in Fig. 1, HTC cells exhibited constitutive ATP release in isotonic buffer, and dilution of media by 20% with water to increase cell volume led to a ~3-fold increase in bioluminescence ($p \leq 0.001$). This response was characteristic of all cell models studied. Addition of NaCl (50 mosmol) to restore tonicity significantly reversed ATP

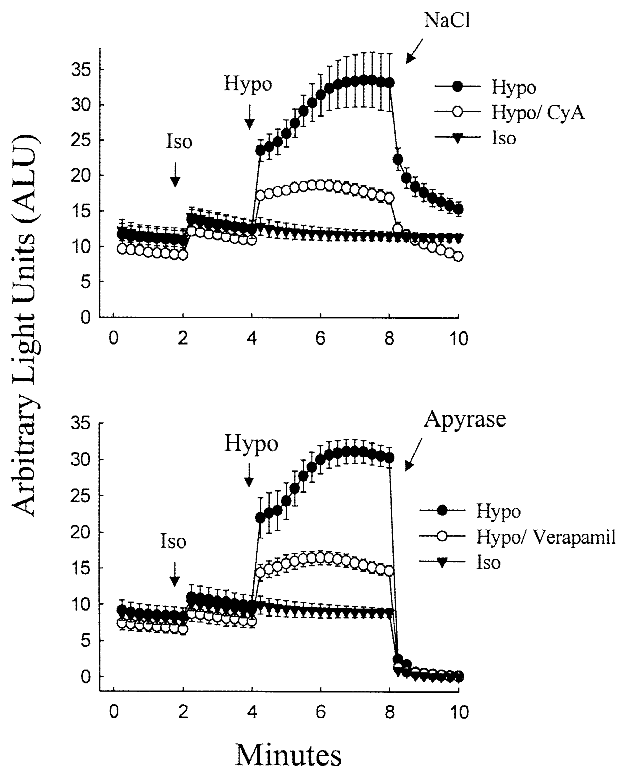

Fig. 1. ATP permeability is sensitive to P-glycoprotein inhibitors. In HTC cells, ATP in the extracellular medium was measured using a luciferase-luciferin assay where an increase in arbitrary light units (*ALU*, y-axis) corresponds to increasing ATP concentrations. Addition of isotonic medium (*Iso, arrow*) resulted in small increases in bioluminescence due to mechanical stimulation. Addition of water to dilute extracellular medium by 20% (*Hypo, arrow*) produced much larger increases due to swelling-induced ATP release. Addition of NaCl (50 mosmol, *upper graph*) to restore tonicity or the ATPase apyrase (1 U/ml, *lower graph*) reversed these changes. Preincubation with P-glycoprotein inhibitors cyclosporin A (10 μM, upper) or verapamil (10 μM, lower) significantly reduced the magnitude of ATP release during hypotonic stress. Values represent means ± SEM; $n = 7$ for each.

permeability. In other studies, addition of the ATPase/ADPase apyrase (1 U/ml) to scavenge extracellular ATP rapidly diminished bioluminescence.

To determine whether P-glycoproteins modulate ATP release, cells were incubated with P-glycoprotein inhibitors for 10 min prior to study. Peak ATP efflux following a 20% media dilution was inhibited 69% and 62% by cyclosporin A (10 μM) and verapamil (10 μM), respectively (each $p \leq 0.001$). Although basal bioluminescence in the absence of hypotonic stress decreased following exposure to both agents, the effects were not statistically significant. These findings suggest that endogenous P-glycoproteins contribute to the regulation of a volume-sensitive ATP-permeability pathway.

Intracellular Perfusion with P-glycoprotein Antibodies Inhibits Volume- but Not ATP-sensitive Cl^- Permeability

In HTC cells, release of ATP into the extracellular space and binding to P2 receptors is critical for swelling-induced Cl^- current activation (Fig. 2) (Wang et al., 1996). Consequently, in single cells the amplitude of volume-sensitive currents serves as a sensitive measure of local ATP concentrations. The monoclonal antibody C219, which recognizes binding regions near the highly conserved cytoplasmic ATP-binding motifs of P-glycoproteins, has been shown to inhibit P-glycoprotein function, presumably by preventing ATP hydrolysis (Vanoye, Altenberg & Reuss, 1997). If P-glycoproteins regulate ATP permeability, then intracellular delivery of C219 antibodies would be expected to decrease activation of Cl^- channels during hypotonic stress. For these studies, the intracellular space of cells undergoing whole-cell patch-clamp recording was dialyzed with C219 antibodies (5 μg/ml final concentration) by inclusion in the patch-pipette solution (Fig. 2), and only cells with a series access resistance ≤10 MΩ were utilized to assure equilibration of the pipette contents with the cell interior. Compared to basal (isotonic) conditions (–1.76 ± 0.48 pA/pF), exposure of control cells to hypotonic buffer (20% reduction in NaCl) led to an increase in outwardly rectifying Cl^- currents that were similar to those previously described (–33.09 ± 12.53 pA/pF at –80 mV, $n = 12$, $p < 0.01$) (Gill et al., 1992). However, intracellular perfusion with the same buffer plus C219 antibodies completely inhibited current activation following hypotonic exposure (–1.96 ± 0.34 pA/pF, $n = 6$, $p < 0.02$). In contrast, the C219 antibodies had no effect on P2 receptor stimulation by exposure to extracellular ATP (10 μM, –65.51 ± 25.80 pA/pF, $n = 4$). The observed inhibition appears specific for C219 binding to P-glycoproteins since cytoplasmic delivery of similar concentrations of heat-inactivated C219 antibodies (100°C for 1 hr, 5 μg/ml) and unrelated β-galactosidase antibodies (5 μg/ml) had no effect on volume-activated currents (–29.30 ± 4.94 and –37.36 ± 3.81 pA/pF, respectively, $n = 4$ for each). This alternative strategy provides additional support for P-glycoprotein-dependent ATP release. Thus, liver P-glycoproteins do not

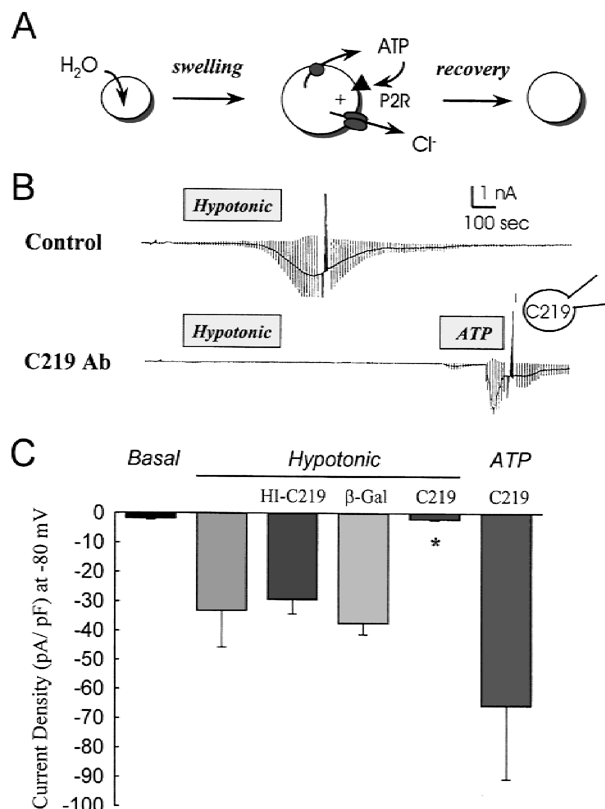

Fig. 2. Intracellular delivery of C-219 antibodies disrupts purinergic signaling. (*A*) In HTC cells, ATP released into the extracellular space activates membrane Cl^- channels coupled to P2 receptors. Cl^- efflux contributes to recovery of cell volume. (*B*) In a representative whole-cell patch-clamp recording, membrane currents were measured at a holding potential of –40 mV and test potentials of 0 mV and –80 mV (400 msec duration at 10 sec intervals). Exposure to hypotonic buffer increased inward currents at –80 mV which correspond to volume-activated Cl^- currents ($I_{Cl\text{-}Swell}$) (Control, *top tracing*). Intracellular dialysis with C219 antibodies (5 μg/ml) to inhibit P-glycoprotein function prevented this response (C219 Ab, *bottom tracing*). However, subsequent exposure to ATP (10 μM) produced a significant increase in currents, indicating that C219 antibodies do not specifically block P2-receptor-activated Cl^- channels. (*C*) Compared to currents (–80 mV) in isotonic buffer (*Basal*), large currents were activated following exposure to hypotonic buffer (*Hypotonic,* $p < 0.01$, $n = 12$). Intracellular dialysis with C219 antibodies significantly inhibited volume-activated currents (C219, $n = 6$), but dialysis with heat-inactivated C219 (HI-C219, $n = 4$) and β-galactosidase antibodies (β-gal, $n = 4$) had no effect. Currents induced by exposure to extracellular ATP (10 μM) in cells perfused with C219 antibodies (*ATP* C219, $n = 4$) were not significantly different from cells not perfused with C219 ($n = 4$, *not shown*). Current is expressed as current density (pA/pF) to normalize for cell size and values represent means ± SEM. Asterisk denotes $p < 0.02$.

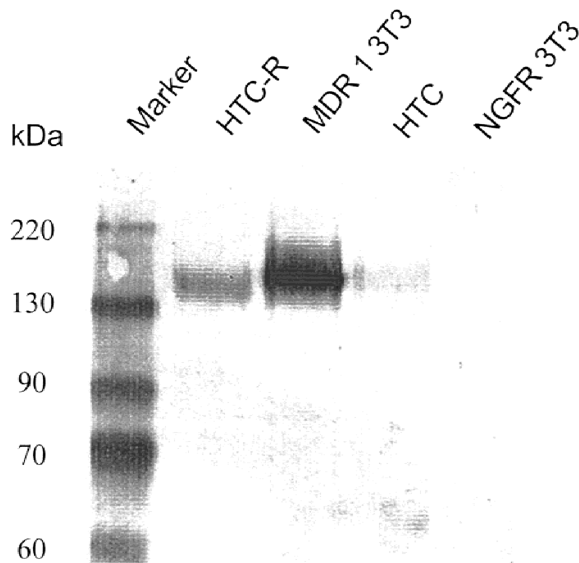

Fig. 3. Western analysis of P-glycoprotein expression. P-glycoprotein expression was determined in cell lysates by Western analysis using C219 antibodies (Methods). HTC-R cells (lane 2) demonstrated increased staining compared to native HTC cells (lane 4), consistent with the upregulation of mdr P-glycoproteins. A marked increase in P-glycoprotein expression was evident in MDR1-transfected (*MDR1 3T3*, lane 3) compared with parental 3T3 cells (lane 5).

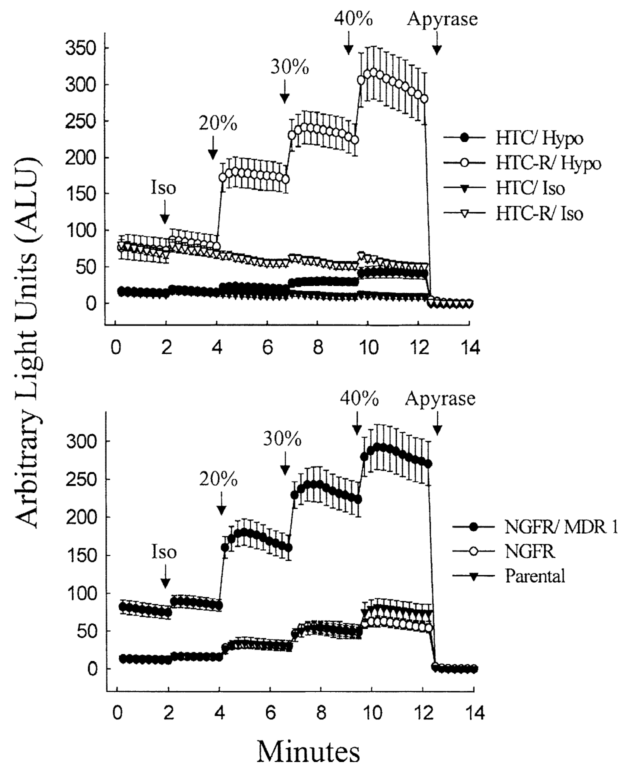

Fig. 4. Expression of P-glycoproteins increases ATP release. The effects of P-glycoprotein overexpression on basal and volume-sensitive ATP release were assessed by bioluminescence. In all studies, bioluminescence increased significantly in response to medium dilution with water to increase cell volume (20–40%, arrows, *Hypo*) compared to controls in which similar volumes of isotonic media were added (*Iso*). (*Top*) Overexpression of mdr proteins in HTC-R cells led to a marked increase in ATP release under basal conditions (*HTC-R/iso*) and during hypotonic stress (HTC-R/Hypo) compared to native HTC cells (*HTC/iso, HTC/hypo*). (*Bottom*) Expression of MDR1 P-glycoproteins in NIH 3T3 cells (*NGFR/MDR1*) also increased constitutive and volume-sensitive ATP release compared to control mock-transfected (*NGFR*) and parental cells. In all studies, addition of the ATPase/ADPase apyrase (*arrow*) eliminated bioluminescence. Values represent means ± SEM; $n = 8$ for each.

appear to interact directly with volume-sensitive Cl^- channels, but rather modulate membrane Cl^- permeability indirectly by regulating a cellular ATP release pathway.

Overexpression of P-glycoproteins Enhances Cellular ATP Permeability

To more specifically assess the role of MDR proteins on cellular ATP permeability, bioluminescence was measured in both rat and human models of MDR overexpression. These include: i) a bile-acid resistant HTC cell clone (HTC-R) which overexpresses multiple mdr proteins compared to HTC cells (Brown et al., 1995), and ii) human NIH 3T3 cells stably transfected with wild-type MDR1, compared to parental and mock-transfected cells. The expression of P-glycoproteins in each of these models was determined by Western blot probed with C219 antibodies (Fig. 3). A low level of staining is evident in HTC cell lysates, which have been shown to express mdr 1b (but not mdr 1a, multidrug resistance protein 2 [MRP2], or sister of P-glycoprotein [sgpg], which are also recognized by C219). In HTC-R cells, C-219 detected a much more prominent band, consistent with increased expression of mdr gene products. As expected, P-glycoprotein expression was greatest in NIH 3T3 cells transfected with MDR1 (~170 kDa), compared to control parental cells, which do not contain endogenous P-glycoprotein.

The luciferase-luciferin assay was utilized to determine the effect of P-glycoprotein expression on cellular ATP efflux, and representative studies are shown in Fig. 4. Compared to HTC cells, ATP release under basal (isotonic) conditions and during dilution of medium to increase cell volume was consistently and markedly increased in HTC-R cells. In a similar fashion, expression of wild-type MDR1 in NIH 3T3 cells (MDR1/NGFR) led to a large increase in ATP permeability compared to mock-transfected (NGFR) and parental control cells. These findings were consistent (>10 experiments for each set), and were not related to differences in cell density (similar mean protein concentrations/plate). For

both control and MDR1-expressing fibroblasts, relative ATP release increased in parallel to graded increases in medium dilutions up to 55%. However, at 13% hypotonicity significant increases in bioluminescence were seen only in MDR1-transfected (69 ± 13% change, $n = 6$, $p < 0.001$) but not in control (parental) cells (22 ± 12% change, $n = 6$, NS), suggesting that P-glycoproteins upregulate the sensitivity of the ATP transport pathway for a given shift in transmembrane osmolar gradient.

A related hepatocyte canalicular ABC protein, the sister of P-glycoprotein (sgpg), has been shown to function as an ATP-dependent bile acid transporter (Gerloff et al., 1998). Sgpg transcripts are undetectable in HTC and HTC-R cells (*data not shown*). In contrast to MDR1, expression of sgpg in human embryonic kidney (HEK) 293 cells did not increase ATP release compared to control (mock-transfected) cells, suggesting that bile acid (sgpg) and nucleotide (MDR) transport pathways are differentially regulated. These findings provide evidence that expression of MDR1 P-glycoproteins in mammalian cell models positively regulates cellular ATP permeability and increases the sensitivity of the ATP transport pathway to hypotonic stress.

The ATP-release Pathway is Distinct from the P-glycoprotein Pump

Regulation of ATP permeability by MDR1 P-glycoproteins could be due either to direct transport of ATP molecules via the P-glycoprotein pump, or, analogous to CFTR, by gating of an associated ATP transport protein. The latter mechanism is supported by the observation that parental NIH 3T3 cells, although they lack P-glycoproteins, also exhibit ATP release. These possibilities were addressed using two experimental approaches as shown in Fig. 5. First, since extracellular $GdCl_3$ is an effective inhibitor of membrane ATP release (Fig. 5*A*), the effect of $GdCl_3$ on efflux of the P-glycoprotein substrate rhodamine-123 efflux was determined (Fig. 5*B*). After loading, NIH 3T3 cells expressing MDR1 demonstrated continuous efflux of rhodamine-123 into medium that was enhanced compared to control NIH 3T3 cells. P-glycoprotein-dependent transport of rhodamine-123 was inhibited by exposure of cells to verapamil (100 μM). In contrast, addition of $GdCl_3$ (200 μM) to media did not change the rate of rhodamine-123 efflux, suggesting that verapamil-sensitive P-glycoprotein and $GdCl_3$-sensitive ATP transport pathways are distinct.

Using a second approach, the effects of alterations in drug transport properties of MDR1 P-glycoproteins on cellular ATP permeability was evaluated. First, ATP release in NIH 3T3 cells expressing V185 mutant and G185 wild-type MDR1 P-glycoproteins was compared. The V185 mutant exhibits altered substrate selectivity, with enhanced and diminished transport of colchicine and vinblastine, respectively, and differs in sensitivity to P-glycoprotein inhibitors (Stein et al., 1994; Choi et al., 1988). Despite these differences in transport characteristics, basal bioluminescence was not significantly different between V185- and G185-expressing cells ($n = 15$ for each). In addition, ATP release in each of these models was >5.5-fold that of parental, P-glycoprotein-deficient controls ($n = 32$, $p < 0.001$, Fig. 5*C*). In other studies, the effect of monoclonal UIC2 antibodies on ATP permeability was assessed. Although UIC2 at concentrations between 5 and 100 μg/ml bind activated P-glycoproteins extracellulary to alter drug transport (Mechetner & Roninson, 1992), addition of these antibodies to extracellular medium (10–200 μg/ml, 10 min preincubation) did not alter basal or hypotonicity-sensitive ATP release in either MDR1/NGFR- or NGFR-expressing fibroblasts. A representative example is shown in Fig. 5*D*, in which exposure of MDR1-transfected and mock-transfected cells to 200 μg/ml of UIC2 antibodies had no effect on ATP permeability. In other studies, addition of control UPC10 antibodies to extracellular medium did not alter detected bioluminescence in these cell models (*data not shown*). Thus, neither a mutation of the transporter pore nor exposure to extracellular inhibitory antibodies that alter drug transport attenuate the ability of human MDR1 P-glycoproteins to increase cellular ATP efflux. Taken together, these findings indicate a clear dissociation between the effects of MDR1 P-glycoproteins on ATP release and substrate transport. Thus, P-glycoproteins are not likely to function as ATP channels, but rather to modulate other cellular ATP-transport pathways.

Discussion

P-glycoproteins have established roles in the transport of a variety of amphipathic compounds, including xenobiotics, carcinogens, and selected drugs (Lum & Gosland, 1995). In addition, the present studies suggest that MDR1 P-glycoproteins are also capable of modulating cell function in an entirely different manner by regulating cellular ATP permeability as originally proposed by Abraham et al. (Abraham et al., 1993). Consequently, MDR1 may serve as a critical regulator of purinergic signaling through effects on ATP release.

In HTC cells, as in primary hepatocytes, increases in cell volume represent a potent stimulus for ATP release, and several observations demonstrate that P-glycoproteins play an important modulatory role in this process. First, volume-sensitive ATP permeability was attenuated by exposure to the putative P-glycoprotein inhibitors verapamil and cyclosporin A. Similarly, intracellular dialysis with C219 antibodies, which have been used in a similar fashion to antagonize P-glycoprotein function, prevented swelling-dependent activation of Cl^- channels,

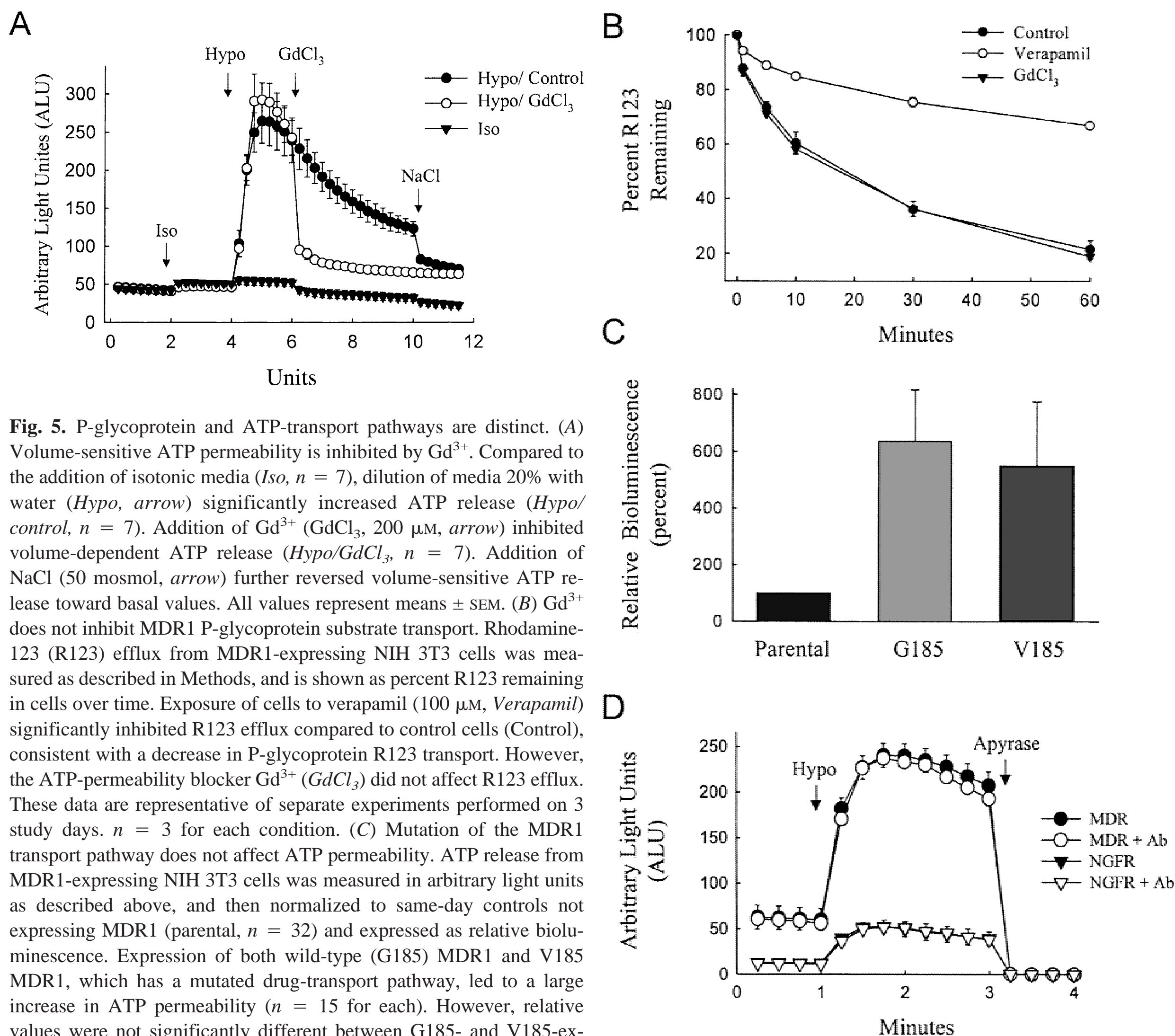

Fig. 5. P-glycoprotein and ATP-transport pathways are distinct. (*A*) Volume-sensitive ATP permeability is inhibited by Gd^{3+}. Compared to the addition of isotonic media (*Iso,* $n = 7$), dilution of media 20% with water (*Hypo, arrow*) significantly increased ATP release (*Hypo/control,* $n = 7$). Addition of Gd^{3+} ($GdCl_3$, 200 μM, *arrow*) inhibited volume-dependent ATP release (*Hypo/GdCl₃,* $n = 7$). Addition of NaCl (50 mosmol, *arrow*) further reversed volume-sensitive ATP release toward basal values. All values represent means ± SEM. (*B*) Gd^{3+} does not inhibit MDR1 P-glycoprotein substrate transport. Rhodamine-123 (R123) efflux from MDR1-expressing NIH 3T3 cells was measured as described in Methods, and is shown as percent R123 remaining in cells over time. Exposure of cells to verapamil (100 μM, *Verapamil*) significantly inhibited R123 efflux compared to control cells (Control), consistent with a decrease in P-glycoprotein R123 transport. However, the ATP-permeability blocker Gd^{3+} (*$GdCl_3$*) did not affect R123 efflux. These data are representative of separate experiments performed on 3 study days. $n = 3$ for each condition. (*C*) Mutation of the MDR1 transport pathway does not affect ATP permeability. ATP release from MDR1-expressing NIH 3T3 cells was measured in arbitrary light units as described above, and then normalized to same-day controls not expressing MDR1 (parental, $n = 32$) and expressed as relative bioluminescence. Expression of both wild-type (G185) MDR1 and V185 MDR1, which has a mutated drug-transport pathway, led to a large increase in ATP permeability ($n = 15$ for each). However, relative values were not significantly different between G185- and V185-expressing cells. (*D*) UIC2 antibodies do not inhibit ATP release. Changes in ATP release following a 20% medium dilution with water (*Hypo, arrow*) in NIH 3T3 cells are shown. Compared to mock-transfected cells (*NGFR,* $n = 6$), basal and volume-sensitive ATP permeability was increased in MDR1-expressing cells (*MDR,* $n = 6$). Addition of UIC2 antibodies (200 μg/ml, 10 min preincubation) did not affect constitutive or volume-sensitive ATP release in MDR1-expressing (*MDR + Ab,* $n = 6$) or control (*NGFR + Ab,* $n = 6$) cells. All values (*A, B, C*) represent means ± SEM.

a biophysical measure of local ATP concentrations (Vanoye et al., 1997). In HTC cells, C219 antibodies are likely to mediate their effects by inhibition of MDR1b P-glycoproteins, since other cross-reacting C219 targets (including MDR1a, multidrug resistance protein 2 and sgpg) are not detectable. Although C219 antibodies have also been reported to cross-react with other proteins such as membrane Cl^- channels in lens fiber cells and zymogen granules (Zhang & Jacob, 1994; Thevenod, Anderie & Schulz, 1994), such channels are not present in HTC cells, and the amplitude of Cl^- currents following P2-receptor stimulation was unaffected by C219 antibodies. Thus, MDR1 proteins present in HTC cells positively regulate membrane ATP transport, which is critical for activation of volume-sensitive Cl^- channels.

Using another approach, the effect of P-glycoprotein overexpression on ATP release was also assessed. In both HTC-R cells, a bile acid-resistant clone of HTC cells that exhibits increased expression of multiple mdr gene products, and NIH 3T3 cells transfected with human MDR1, there were substantial increases in constitutive and volume-sensitive ATP permeability. In the latter model, the effects were selective and specific for MDR1, and were not observed after overexpression of a related canalicular bile-acid transport protein sgpg (Gerloff et al., 1998). Since MDR1 P-glycoproteins and sgpg are both expressed in the canalicular membrane of hepatocytes, the transport of bile acids (sgpg) and ATP (MDR1) into bile appears to be differentially regulated. This finding may have physiological importance, as ATP

is present in physiological concentrations in human bile and has been implicated in the regulation of transepithelial biliary secretion and bile formation (Roman & Fitz, 1999).

It is notable that all cells tested exhibited ATP release, even those without detectable P-glycoproteins. In the presence of MDR1, P-glycoprotein-dependent transport of rhodamine-123 was unaffected by a potent inhibitor of ATP efflux, $GdCl_3$. In addition, neither mutations that alter P-glycoprotein substrate specificity (V185) nor exposure to monoclonal antibodies that recognize a functional conformation on P-glycoproteins and block P-glycoprotein-mediated drug transport (UIC2) had any effect on ATP release. Thus, P-glycoprotein substrate transport can be dissociated from ATP release. Taken together, these findings suggest that MDR1 P-glycoproteins are probably not directly transporting ATP but rather function to modulate neighboring ATP release proteins that are present in the absence of P-glycoprotein expression. This paradigm appears similar to emerging evidence for the ability of ABC transporters to regulate the function of other membrane-associated proteins (Clement et al., 1997).

Despite the potential implications of these experiments, interpretation is limited by important unknown variables. First and most significantly, the proteins (channels, transporters) that mediate epithelial ATP permeability have not been identified. Although anionic ATP molecules have been shown to permeate membrane channel pores, the physiological contribution of these versus other ATP-release pathways (e.g., quantal vesicular efflux) will require cloning and characterization of specific ATP-transport proteins. As a result, the mechanistic relationships between P-glycoproteins (or other ABC members) and ATP-transport proteins remains undefined. Second, there is an apparent discrepancy between inhibitory effects of "chemosensitizers" on ATP release compared to the noninhibitory effects of mutational and antibody-dependent manipulations of P-glycoprotein transport. Although verapamil and cyclosporin A attenuate substrate transport, they have also been shown to alter P-glycoprotein (e.g., modulation of ATPase activity) and cell function as well. Due to the potential nonspecific effects of these agents, findings in HTC cells were supported by more targeted inhibition of native P-glycoproteins via delivery of C219 antibodies to the cell interior, supporting a specific role for P-glycoproteins in modulation of ATP release. Finally, since ABC protein expression is limited to certain cell types, there must be alternative regulatory pathways that modulate ATP permeability in other tissues. In that light, caution is warranted in the application of these findings in HTC cells to intact liver where the regulatory environment may be distinct.

Taken together, these findings support a specific and quantitatively important role for P-glycoproteins encoded by the MDR1 gene in the regulation of both constitutive and volume-sensitive ATP release. P-glycoproteins do not appear to transport ATP molecules directly, but rather to regulate a separate membrane-associated ATP transporter. Characterization of the relationship between P-glycoproteins expression and cellular ATP release pathways may provide new strategies for modulation of extracellular purinergic signaling through regulated nucleotide release.

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