Published in final edited form as:
Annu Rev Pharmacol Toxicol. 2013 ; 53: 231–253. doi:10.1146/annurev-pharmtox-010611-134609.

Cyclic Nucleotide Compartmentalization: Contributions of Phosphodiesterases and ATP-Binding Cassette Transporters

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Abstract

Cyclic nucleotides [e.g., cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP)] are ubiquitous second messengers that affect multiple cell functions from maturation of the egg to cell division, growth, differentiation, and death. The concentration of cAMP can be regulated by processes within membrane domains (local regulation) as well as throughout a cell (global regulation). The phosphodiesterases (PDEs) that degrade cAMP have well-known roles in both these processes. It has recently been discovered that ATP-binding cassette (ABC) transporters contribute to both local and global regulation of cAMP. This regulation may require the formation of macromolecular complexes. Some of these transporters are ubiquitously expressed, whereas others are more tissue restricted. Because some PDE inhibitors are also ABC transporter inhibitors, it is conceivable that the therapeutic benefits of their use result from the combined inhibition of both PDEs and ABC transporters. Deciphering the individual contributions of PDEs and ABC transporters to such drug effects may lead to improved therapeutic benefits.

Keywords

CFTR; cAMP; efflux; export; MRP4

EARLY EVIENCE FOR CYCLIC NUCLEOTIDE COMPARTMENTALIZATION

The first evidence for a nonuniform cellular effect of increases in cAMP was provided by the work of Buxton & Brunton (1), who compared cAMP activation of protein kinase A

DISCLOSURE STATEMENT

J.-S.H. holds a patent for inhibitors of MRP4 for the treatment or the prevention of vascular disorders (US 2010/0278809 A1) and has received funding from Agence Nationale de le Recherche (09JCJC0112) and Fondation de France (2006005606). The other authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

(PKA) in response to treatment of perfused rabbit heart with agonists of different G protein–coupled receptors (GPCRs): prostaglandin E_1 (PGE_1, a prostanoid receptor agonist) or isoproterenol (a β-adrenergic receptor agonist). After treatment, the heart was homogenized and separated into soluble and particulate fractions, and the amount of PKA in each fraction was determined. This analysis revealed that the ratio of activated PKA in soluble fractions to activated PKA in particulate fractions was ligand dependent. Notably, PGE_1 increased the activity primarily of the soluble PKA, whereas isoproterenol increased particulate PKA; moreover, the increase in particulate, but not soluble, PKA activity was associated with positive cardiac inotropy. These studies (1) suggested that after its formation, the "cAMP message" was compartmentalized and might produce different biological effects if cAMP signals were spatially segregated within a cell.

An elegant series of experiments by Jurevicius & Fischmeister (2) provided evidence for such spatial regulation. The authors used patch clamps to record cAMP-activated calcium currents (L-type Ca2C) from two physically separated sites on a single isolated cardiac myocyte after β-adrenergic receptor stimulation. These investigators hypothesized that if cAMP activation occurs throughout the cell, then locally applied adrenergic stimulation would activate calcium currents at both sites. They found that local application of isoproterenol produced a restricted activation of calcium currents (i.e., compartmentalized effect), whereas local application of forskolin (a general adenylyl cyclase activator) activated the calcium current throughout the cell (i.e., produced global effects). Many factors contribute to cAMP compartmentalization, but here we primarily describe recent evidence for the role of ATP-binding cassette (ABC) transporters in the process. We relate this role to that of phosphodiesterases (PDEs) because they are important therapeutic targets, and some PDE inhibitors might exert part of their effect owing to inhibition of ABC transporters.

PHOSPHODIESTERASES MODULATE LOCAL cAMP CONCENTRATION

Activation of PKA requires the binding of cyclic adenosine monophosphate (cAMP) to the PKA holoenzyme, which produces dissociation of its regulatory and catalytic subunits. However, to elicit PKA activation, cellular cAMP concentrations need to increase in excess of the K_m of PKA [~3 μM (3)]. Because cAMP has a high diffusion rate (500–700 $\mu m\ s^{-1}$), PKA activation could theoretically occur throughout a cell by any stimulus that elevated intracellular cAMP. This leads to the question: How is PKA specifically activated in response to receptor-mediated elevation in cAMP? In other words, what prevents cAMP, once formed, from always producing global activation of both the soluble and particulate forms of PKA?

The limitation of PKA activation by freely diffusible cAMP may occur via physical barriers (e.g., endoplasmic reticulum below the plasma membrane or membrane invaginations) or enzymatic barriers that restrict intracellular diffusion of cAMP (4, 5, 6, 7). An enzymatic barrier appears to be provided by PDEs. Cyclic nucleotide PDEs constitute a group of enzymes responsible for the degradation of the phosphodiester bond in cAMP and cyclic guanosine monophosphate (cGMP); this degradation results in the hydrolysis of cAMP and cGMP and the formation of adenosine 5′-monophosphate (AMP) and guanine 5′-

monophosphate (GMP), respectively. PDEs localize either to the cytosol or to the subcellular compartments, including the plasma membrane, but they can be recruited into multiprotein signaling complexes (8). The amino terminal sequence of a PDE can determine its subcellular localization, and such localization appears to determine whether a PDE is activated.

Eleven mammalian PDE gene families containing 21 genes (9, 10) have been described. The families are clustered according to criteria that include structural similarity, sequence homology, protein domains, and various enzymatic properties such as sensitivity to endogenous regulators and inhibitors, kinetic properties, and substrate specificity. PDEs are grouped into three categories with respect to their substrate specificities: cAMP-specific (PDE4, PDE7, and PDE8), cGMP-specific (PDE5, PDE6, and PDE9), and dual-specific (PDE1, PDE2, PDE3, PDE10, and PDE11) (4). This review focuses on several PDEs that have well-defined roles in compartmental signaling.

Brechler et al. (11) provided evidence for a role of PDEs in compartmentalized cAMP signaling that is initiated by the hormone glucagon in heart cells. Prior studies had shown that glucagon increased cAMP, leading to activation of calcium channels (12). However, glucagon did not activate adenylyl cyclase; instead, by acting through a pertussis toxin–sensitive pathway, it inhibited a membrane-bound PDE that has a high affinity for cAMP. The increase in cAMP was not attributable to inhibition of a soluble form of PDE. Through the judicious use of PDE inhibitors, the authors suggested that an elevation of cAMP in the membrane contributed to the positive inotropic effect of glucagon. Because pharmacologic inhibition of PDE affects cAMP concentrations in specific subcellular compartments, considerable effort has been directed toward understanding how compartmentalized signaling works, with cAMP degradation by PDEs underpinning the mechanism.

Recent studies that utilize live-cell imaging and florescence resonance energy transfer (FRET) have provided strong support for the idea that PDEs have a role in compartmentalized cAMP signaling. Experiments performed by Leroy et al. (13) revealed the contribution of PDEs to the intracellular spatiotemporal dynamics of cAMP concentration in ventricular myocytes. By using pulse treatments of a β-adrenergic receptor agonist, these authors showed that cAMP peaks are observed faster at the membrane than in the cytosol. This finding suggests that cAMP is rapidly synthesized and hydrolyzed at the plasma membrane and that diffusion is restricted in the cytoplasm (13). Interestingly, Nikolaev et al. (14) showed that localized β_1-adrenergic receptor stimulation generates a cAMP gradient that propagates throughout the cells, whereas localized β_2-adrenergic receptor stimulation does not elicit cAMP diffusion. If the cAMP spatiotemporal dynamics depends on the source of cAMP, then the contributors to compartmentalization may be part of a specific organization. Additional studies using FRET technology showed that two different PDE4 subfamilies, PDE4B and PDE4D, are responsible for cAMP dynamics in defined compartments of the cell (Figure 1). Results from this study suggest that PDE4B regulates a subplasma membrane cAMP compartment, whereas PDE4D contributes mainly to cytosolic cAMP regulation. This finding led to a model in which cytosolic PDE4D acts as a sink to locally drain cAMP (15).

The concept that PDE4D controls localized cAMP concentration suggests the possibility that multiple cAMP gradients can be simultaneously generated in different cellular locations. Because of the rapid diffusion of cAMP (see above), this gradient would depend not on the distance from adenylyl cyclase but instead on its proximity to the closest PDE4 sink. Localized pools of PDE4 might then restrict inappropriate, excess activation of cAMP effectors such as PKA. This concept of PDE sinks is supported by studies using FRET reporters fused to individual PDEs; such studies reveal the formation of cAMP gradients around PDE molecules (15). To further explore intracellular cAMP gradients, investigators modified the cAMP binding protein, Epac (Exchange protein activated by cAMP), as a FRET reporter for cAMP (CFP-Epac-YFP, with CFP fused to the N terminus and YFP to the C terminus). HEK293 cells were transfected with CFP-Epac-YFP and then treated with a β-adrenergic receptor agonist to increase cAMP production in the presence and absence of PDE4 inhibitors, rolipram, and Ro20-1724. Cells treated with either of these two compounds produced a rapid decrease in the YFP-FRET, indicating an increase in intracellular cAMP levels upon inhibition of PDE4. The authors demonstrated the selective nature of this effect by using PDE3 and PDE5 inhibitors, which produced no change in FRET (15, 16). These results demonstrated that specific PDEs regulate cAMP concentrations and that this regulation depends on the location of the PDEs.

PDEs are located in multiple subcellular compartments, and putative or established targeting domains have been identified for most of the PDE families (17). PDE3s target the endoplasmic reticulum by a transmembrane domain consisting of six transmembrane helices (18), and PDE4D5 interacts with RACK-1, a scaffolding protein that binds certain PKC isoforms after their activation by diacylglycerol (19). PDE4D3 is targeted to the Golgi/centrosomal region through anchoring by myomegalin (20). Some PDE4D and PDE4A variants bind Src homology 3 domains of, e.g., Src kinases (21–23); furthermore, PDE4 isoforms, via their catalytic domain, bind to and are phospho-rylated by Erk (24). PDE4A1 contains a novel lipid binding domain, TAPAS, that has specificity for phosphatidic acid and that serves to target this PDE to specific cellular membranes (25).

PHOSPHODIESTERASES CLUSTER IN CAVEOLAE

Whereas PDEs reside in various subcellular compartments, such as the plasma membrane, cytoplasm, and subnuclear areas, they may also cluster in domains that modulate multiple incoming cAMP signals. Plasma membrane caveolae are one type of structure in which PDEs are found. Caveolae (“little caves”), flask-like invaginations of the plasma membrane, are proposed to be organizers of signal transduction in numerous cell types, in particular pulmonary vascular endothelial cells (26, 27). Caveolae are morphologically distinct entities that constitute a subset of lipid rafts that organize membrane lipid and protein components. In addition, they are enriched with particular lipids (e.g., cholesterol, glycosphingolipids) and scaffolding proteins (e.g., caveolins) that interact with multiple proteins (28).

One might expect that some PDEs would localize to caveolae as a means to modulate membrane changes in cAMP concentration. Indeed, PDE3B localizes to caveolae in primary adipocytes, as demonstrated by coimmunoprecipitation with caveolin-1 (29). This physical interaction appears to play a regulatory role because disruption of caveolae with methyl-β-

cyclodextrin (by removing cholesterol from the membrane) reduces PDE3B expression. PDE3B expression is also reduced in caveolin$^{-/-}$ mice, as shown by Nilsson et al. (29). The authors proposed that caveolin-1 is required to stabilize PDE3B. However, not all PDEs are stabilized by caveolin-1. For instance, the relationship between PDE5 and caveolin-1 in pulmonary artery smooth muscle cells shows that when caveolin-1 is overexpressed, PDE5 expression is decreased; Murray et al. (30) speculated that this inverse relationship arose not from a direct interaction but from an undefined mechanism. Further knowledge of the interactions of PDEs and caveolin is necessary to fully define the contribution of caveolae to cAMP compartmentalization.

PHOSPHODIESTERASE INHIBITORS

Multiple PDE inhibitors have been developed as therapies for pathological conditions that might be modulated by altering cyclic nucleotide levels. PDEs are good therapeutic targets because of their high affinity for substrates and unique substrate binding requirements that make them amenable to small-molecule inhibitors (31). However, because overall concentrations of cAMP and cGMP in most cell types range from <1 μM to ~10 μM, developing competitive inhibitors that block PDE activity is a challenge. Nonetheless, pharmacologic inhibition of PDEs has been achieved and used to support the idea of cAMP compartmentalization.

The location of a PDE within a cell might impact the efficacy of a PDE inhibitor (Figure 1). For example, within the PDE3 subfamily, PDE3A is either membrane associated or cytosolic, whereas PDE3B is predominantly membrane associated. Several isoform-selective PDE3 inhibitors such as amrinone, milrinone, cilostamide, cilostazol, and trequinsin are available, but none of these compounds distinguish between PDE3A and PDE3B. One compound, OPC-33450, shows selectivity between the isoforms (32). Both PDE3A and PDE3B are expressed in vascular smooth muscle cells and regulate vascular contractility (33). Recent studies found that PDE3A is expressed at the apical plasma membrane of epithelial cells, where it interacts directly with the cystic fibrosis transmembrane conductance regulator (CFTR) channel. Notably, PDE3 inhibition by cilostazol generated compartmentalized cAMP, which potentiated CFTR channel function. These interactions required an intact actin cytoskeleton because the actin polymerization inhibitor latrunculin B disrupted both the PDE3A-CFTR interaction and the compartmentalization of cAMP (34).

PHOSPHODIESTERASE RECRUITMENT

PDEs are vital to maintaining proper cAMP concentration within a cell. Knockout models of PDE4B (31) have illustrated how signaling processes that regulate biological processes are altered. If a cell lacks PDEs, it might become overrun by cAMP and produce promiscuous activation of multiple cAMP-dependent pathways. The close proximity of PDEs to the site of cAMP formation constrains cAMP dissemination throughout a cell. Thus, compartmentalized PDEs produce low local concentrations of cAMP (Figure 1), and PDEs can be recruited to protein complexes to attenuate cAMP signaling.

A well-characterized example of PDE recruitment is the relationship among β_2-adrenergic receptor (β_2AR), β-arrestin, and PDE4. In a study performed on HEK293 cells, agonist stimulation of β_2AR increased cAMP concentration and activated PKA. Activated PKA, in turn, phosphory-lated β_2AR, which recruited β-arrestin to PDE4 in close proximity to the β_2AR. This attenuated the local cAMP concentration and, concurrently, abrogated further phosphorylation of the β_2AR by PKA (35, 36). This interaction provides a mechanism whereby cAMP-degrading enzymes localize close to the origin of cAMP synthesis in an agonist-dependent fashion.

MEMBRANE EFFLUX TRANSPORTERS IN INTRACELLULAR CYCLIC NUCLEOTIDE HOMEOSTASIS

Although regulation of cyclic nucleotides occurs by PDEs, other processes can also alter the intracellular cyclic nucleotide concentration. Export of monophosphorylated nucleotides was first noted by Davoren and Sutherland (37, 38), who discovered the biosynthesis of cAMP in response to hormonal stimulation and adenylyl cyclase activation. The authors observed not only an increase in intracellular cAMP but also a concurrent rise in extracellular cAMP. This finding was reported in the 1960s (37). Subsequent studies conducted in the 1970s (39, 40) showed that nucleoside monophosphate analogs exhibit similar export properties, suggesting a common mediator. Many different types of cells export cAMP, including erythrocytes, hepatocytes, endothelial and epithelial cells, neuronal cells, and fibroblasts. However, the ability to export cAMP varies among cell types. Some cells have first-order elimination-rate constants for cAMP that range from 0.14 min^{-1} to 0.014 min^{-1}, corresponding to a half-life of intracellular cAMP from a few minutes to almost one hour. This range could be due to differences in the amount of transporter or perhaps the presence of multiple cAMP export transporters (see below). Furthermore, this nucleotide extrusion process has several general properties, such as being unidirectional, inhibited by depletion of cellular energy [i.e., adenosine triphosphate (ATP)], and chemically inhibited by compounds such as probenecid (an organic anion transport inhibitor) and prostaglandins, as well as having saturable kinetics (41–44).

Akin to cAMP, cGMP is another natural nucleotide that undergoes active efflux from cells. For example, isolated rat hepatocytes stimulated with cytokines and lipopolysaccharide produce nitric oxide (which activates guanylyl cyclase to produce cGMP), but only a small elevation occurs in intracellular cGMP concentration compared with a large increase in extracellular cGMP levels. As observed for cAMP extrusion, cGMP export is inhibited by probenecid (45). Further studies have demonstrated that cGMP efflux is not unique to the liver or kidney; it also occurs in other cells such as vascular smooth muscle cells, endothelial cells, and fibroblasts (46).

In 1999, investigators identified a transporter termed multidrug resistance protein 4 (MRP4; also known as ABCC4), a member of the ABC transporter family (see Mechanism of ATP-Binding Cassette Transporters and Substrate Recognition, below). MRP4 can export nucleotide analogs such as a purine nucleotide monophosphate analog that has antiviral properties (47). The resemblance of this substrate to cAMP prompted subsequent studies to evaluate if MRP4 was capable of ATP-dependent transport of cAMP and cGMP (Figure 2).

Two approaches were used: membrane vesicle transport systems and mammalian cells engineered to stably overexpress MRP4. MRP4-dependent transport activity was assayed through the employment of purified, everted membrane vesicles prepared from insect cells programmed to express MRP4. The everted MRP4-containing vesicles allowed determination of the kinetic properties and ATP dependence of transport. These studies showed that vesicular uptake of cAMP and cGMP requires ATP. Moreover, collapse of the osmotic gradient disrupted transport, confirming that the cyclic nucleotide was adsorbing to the surface of the vesicle not in a nonspecific fashion but instead, in an ATP-dependent fashion (48, 49). These initial studies indicated that the affinity constants of MRP4 for cAMP and cGMP are 45 μM and 10 μM, respectively; however, subsequent studies using other systems have suggested that the K_m for cyclic nucleotide transport is in the 0.5-mM range (50). The reason for the discrepancy is unclear. Nonetheless, these findings provided a mechanism to account for the energy-dependent export of cyclic nucleotides from cells originally observed by Davoren & Sutherland (37).

MECHANISM OF ATP-BINDING CASSETTE TRANSPORTERS AND SUBSTRATE RECOGNITION

ABC transporters mediate the ATP-dependent extrusion of a diverse array of endogenous and exogenous substances (52). These transporters are phylogenetically conserved and expressed in microbial pathogens, plants, and higher organisms. There are 48 transporters belonging to the ABC superfamily in the human genome. Defects in certain genes are the basis of inherited diseases that include a neonatal surfactant deficiency (53), a bleeding disorder and macular degeneration (54), and liver diseases (55), all of which are caused by the failure to export a specific ligand across a lipid bilayer (52). Some human ABC transporters also appear to function in a protective capacity by exporting cytotoxic compounds (e.g., dietary cytotoxics and therapeutic drugs) out of cells. These transporters [e.g., P-glycoprotein (ABCB1), BCRP (ABCG2), and MRP1 (ABCC1)] are highly expressed in the gut, liver, and kidneys, where they also restrict the bioavailability of administered drugs (56–58).

In the ABC transporter superfamily, ABC exporters can be distinguished from ABC importers by the directionality of transport and the distinct structural arrangements of the membrane-spanning domains (MSDs) (Figure 3). All ABC transporters contain two nucleotide-binding domains (NBDs), each of which carries a canonical ABC motif and two MSDs; each of these MSDs usually contains six transmembrane helices in exporters. The MSDs are not highly conserved and form the ligand binding sites to provide substrate specificity. The NBDs are highly homologous throughout the ABC superfamily and have several characteristic motifs, including the Walker A and B motifs common to many nucleotide-binding proteins (59). However, other motifs, such as the ABC signature and the D, H, and Q loops, are unique to the family (60). Interestingly, bacterial and archaeal ABC transporters are typically expressed as half-transporters, with one NBD and one MSD on a single polypeptide chain. The two polypeptides then assemble into a functional homo-or heterodimer. In eukaryotes, however, ABC exporters are often expressed as a single polypeptide chain containing all four domains (two NBDs and two MSDs) (61).

The concept of export of drugs was first proposed by Dano (62) as a mechanism of cancer chemotherapeutic drug resistance. The multidrug resistance P-glycoprotein (ABCB1) was first characterized by Juliano & Ling (63) in the 1970s and was cloned in the 1980s. In 1992, Cole, Deeley, and colleagues (64) cloned the multidrug resistance-associated protein gene, now known as *ABCC1* or *MRP1*. The expansion of the human ABC family accelerated in the 1990s by the expressed sequence tag (est) database, a collection of DNA sequence information from cDNA libraries, and subsequent efforts using computer-aided data mining to identify homologous genes. These tools allowed investigators to expand the members of the ABC superfamily.

In general, the NBDs of ABC transporters bind ATP and hydrolyze ATP (there is no evidence for autophosphorylation of the transporter). Current models indicate that vectorial transport of substrates is provided by ATP binding and hydrolysis (52, 65). Substrate transport occurs against a transmembrane concentration gradient for hydrophilic substrates and against the lipid-water partition coefficient for hydrophobic substrates. In general, the affinity of the MSDs for the ligand is coupled to ATP binding to the NBDs: In the absence of ATP, the MSDs have high affinity for ligand, whereas in the presence of ATP, the affinity is reduced. More detailed models have been proposed to explain this catalytic cycle of energetic coupling between the NBDs and the transport by the MSDs (52).

ABCC4/MRP4 AS A REGULATOR OF cAMP

The domain organization of MRP4 is typical of ABC transporters: The core structure is composed of two MSDs, each of which consists of six transmembrane helices and two cytosolic ATP-binding domains, which bind and hydrolyze ATP to power substrate transport. MRP4 is ubiquitously expressed in many tissues, including the prostate, liver, testis, ovary, and kidney (66); the blood-brain barrier (67); the cardiovascular cells (see Transporters as Regulators in Disease, below); the adrenal gland (48); and the Leydig cells, where it has a role in regulating cAMP-activated de novo testosterone biosynthesis (68). Among the ABCC subfamily, MRP4 is unique, because in polarized cells it can localize either to the apical or basolateral membrane (67). Recent studies have proposed that interactions through MRP4's C-terminal PDZ interaction motif contribute to its polarized expression in certain cells (69, 70).

A biological role for MRP4 in regulating cAMP export was described by studies on a gut epithelial cell line, HT29-CL19A, which expresses both MRP4 and CFTR on the apical plasma membrane (71). These polarized cells vectorially transport cAMP across the apical membrane. This transport is inhibited by the MRP4 inhibitor MK571 or the MRP4 substrate PMEA [9-(2-phosphonyl methoxyethyl)adenine], demonstrating that MRP4 functions as a cAMP exporter. Because the CFTR channel is activated by cAMP, inhibition of MRP4 transport activity potentiates the cAMP-activated CFTR channel in these cells. Whereas MRP4 can export cAMP, it appears that MRP4 also regulates the CFTR channel by modulating cAMP in a compartmentalized fashion. The potentiation of the CFTR channel by either inhibition or silencing of MRP4 was most prominent in response to treatment with low concentrations (<20 μM) of the cAMP-elevating agent adenosine but not with higher concentrations (>20 μM). Real-time monitoring of cAMP dynamics (using the

aforementioned CFP-Epac-YFP) in response to adenosine and another cAMP-elevating agent, forskolin, demonstrated that MRP4 inhibition induces localized cAMP accumulation near the plasma membrane. In addition, low concentrations (2 μM) of adenosine produce a further increase in cAMP concentration near the plasma membrane. This increase revealed how MRP4 regulates cAMP levels in a compartmentalized fashion. However, MRP4 inhibition failed to show local cAMP effects at adenosine concentrations that strongly elevate intracellular cAMP concentrations, suggesting that compartmentalization is overridden at high intracellular cAMP concentrations. Nonetheless, MRP4 can regulate CFTR at low adenosine concentrations by the physical association of MRP4 and CFTR through the PDZ-binding protein PDZK1. [MRP4 has a conserved C-terminal PDZ motif (ETAL).] How the formation of this macromolecular complex is regulated is unknown, but its components may exist within larger domains. In this respect, it is notable that a recent study found that MRP4 localizes in caveolin-enriched fractions (72). Because caveolins are integral membrane proteins that act as a scaffold for membrane proteins in microdomains, it is conceivable that caveolins physically anchor MRP4 in microdomains.

Recently, Sellers et al. (73) determined that MRP4 is expressed in mouse ventricular myocytes. Using a cAMP FRET reporter, they found that MRP4 has a role in β-adrenergic-receptor-stimulated contraction because MRP4 inhibition potentiates submaximal (but not maximal) isoproterenol-stimulated cAMP accumulation and contraction rate. One notable finding was the demonstration that MRP4-dependent regulation of myocyte contraction rate was CFTR dependent, whereas PDE4-dependent potentiation of contraction rate was CFTR independent. The study suggests that PDE4 and MRP4 use independent processes to regulate localized cAMP levels. Moreover, these studies suggest that MRP4 function might be modulated through the formation of a macromolecular complex with CFTR.

Akin to what occurs with certain PDEs, MRP4 promotes the modulation of local membrane cAMP concentrations that are coupled to some GPCR-mediated events. To accomplish local regulation of cAMP, MRP4 forms macromolecular complexes in specialized subcellular domains (see Transporters as Regulators in Disease, below).

OTHER ABC TRANSPORTERS CAPABLE OF EXPORTING CYCLIC NUCLEOTIDES

Additional ABC transporters have been shown to export cyclic nucleotides (Figure 4 and Table 1). For instance, Jedlitschky et al. (74) used membrane vesicles to show that a close relative of MRP4, MRP5 (ABCC5), transports cGMP with micromolar affinity (and cAMP with much lower affinity). Studies using intact cells showed that MRP5 was capable of exporting cAMP from cells engineered to overexpress MRP5 at rates greater than the export rates from unmodified cells and in an ATP-dependent fashion (50).

These studies on intact cells suggest that MRP4 and MRP5 are low-affinity cyclic nucleotide exporters that may function as overflow pumps when phosphodiesterases are limiting (50). In the absence of adenylyl cyclase activation, inhibition of MRP4 transport does not lead to substantial increases in intracellular cyclic nucleotide concentrations, and overexpression of MRP4 does not lead to a substantial decrease in intracellular cAMP levels, even when the

total cAMP concentrations are high (50). These findings fostered the idea that MRP4 is a cAMP overflow pump. However, this idea arose prior to the demonstration that MRP4 modulated local membrane concentrations of cAMP (71). Regulation by MRP4 of local and global cAMP concentrations may depend on the amount of transporter at the plasma membrane, and MRP4 may remain in a nonfunctional state prior to changes in cAMP concentration that occur after adenylyl cyclase activation. Recent studies with sea urchins revealed that although the total amount of an ABC transporter is constant, the functional amount in the plasma membrane is regulated by engagement with the cytoskeleton (75).

At least two other ABC transporters have been shown to export cyclic nucleotides: ABCG2 and ABCC11 (MRP8). MRP8, like MRP4 and MRP5, lacks the third N-terminal MSD found in MRP1, MRP2, MRP3, MRP6, and MRP7. Overexpression of MRP8 in an LLC-PK1 pig kidney cell line led to the following result: Upon adenylyl cyclase activation, intracellular cAMP was reduced in MRP8-overexpressing cells, and cAMP export was enhanced. Notably, an MRP8 loss-of-function allele determines the dry earwax phenotype that is frequently found in East Asians. However, because earwax contains many aliphatic compounds that are potential substrates, it is unlikely that a cyclic nucleotide is its endogenous substrate.

In addition to its role in transporting chemotherapy drugs (76), ABCG2 also serves as a progenitor cell marker (77). Although cyclic nucleotides can impact progenitor cell differentiation (78, 79), whether ABCG2 is a cAMP transporter is not known. However, utilizing combinations of knockout mice, Borst and colleagues (80) demonstrated that Abcg2 and Abcc4 (Mrp4) are the predominant cGMP transporters in murine erythrocytes, whereas Mrp5 has no obvious role in erythrocyte cGMP transport. Because Abcg2 exhibits additional functional overlap with Abcc4 [it transports similar chemotherapeutic nucleotide analogs (81)], it is possible that one endogenous role is the transport of cAMP.

The ABC transporters ABCC4, ABCC5, ABCC11, and ABCG2 are widely expressed in human tissues (Figure 5). ABCC5 is highly expressed in multiple areas in the brain. ABCG2 is highly expressed in the intestine and placenta, unlike ABCC4 and ABCC5, which are expressed at low levels. One could infer from these findings that regulation of cyclic nucleotides by ABC transporters in certain tissues depends on both the amount of transporter and the number of different types of ABC transporters present. Perhaps future studies on tissues from knockout animals will reveal these roles.

INTERACTIONS BETWEEN PHOSPHODIESTERASES AND MRP4

Prior to the identification of MRP4 as a transporter that can efflux intracellular monophosphory-lated nucleotides, some cell types were noted to export a substantial portion of their intracellular cAMP, whereas others exported much less and appeared to rely on PDEs to modulate intracellular cAMP concentrations (82). In certain cell culture systems, such as fibroblasts (83) and smooth muscle cells (84), inhibiting cAMP efflux by a nonspecific organic anion inhibitor (probenecid) did not elevate intracellular cAMP levels compared with untreated cells. From these studies, it was concluded that PDEs have a major role in regulating intracellular cyclic nucleotide concentrations. In contrast, in avian and

mammalian erythrocytes, cyclic nucleotide export was the predominant mechanism regulating intracellular cyclic nucleotide concentrations (40, 85). Furthermore, in mammalian reticulocytes, β-adrenergic receptor activation was associated with cAMP export. Export could be inhibited by ATP-depleting agents (iodoacetate and dinitrophenol) as well as by probenecid and prostaglandin A_1 (40). Moreover, this cyclic nucleotide egress occurred even after cessation of adenylyl cyclase stimulation and was dependent only on the availability of intracellular cAMP.

Some PDE inhibitors are effective inhibitors of MRP4 and MRP5 transport (86) (Table 2). This raises the possibility that the therapeutic benefit and/or untoward side effects might be attributable to MRP4 inhibition as well as PDE inhibition. Jedlitschky et al. (74), using membrane vesicles, found that PDE inhibitors potently inhibit MRP5 transport function. A subsequent study found that the PDE inhibitors trequinsin, zaprinast, and sildenafil inhibit transport by MRP4 (87). Among the PDEs that inhibit MRP4, trequinsin was reported to be the most potent inhibitor (87). The concentrations producing 50% inhibition of MRP4 by these PDE inhibitors are in the low micromolar range (Table 2), which, for some of these compounds, is comparable with the concentration required for PDE inhibition. Interestingly, an estimate of the typical therapeutic dose for the PDE inhibitor sildenafil (~3 μM) suggests a disconnect between the dose required to inhibit its target PDE, PDE5, in vitro (3–350 nM) and therapeutic efficacy. Thus, it is possible that MRP4 inhibition in vivo accounts for some of its therapeutic effect. Furthermore, our recent studies show that low concentrations of cilostazol increase cAMP concentrations close to the plasma membrane (34). This is consistent with our findings demonstrating that this compound is a potent Mrp4 inhibitor (S. Cheepala & J.D. Schuetz, unpublished data). Reciprocally, a recent study reported that treatment with sildenafil led to a progressive increase in MRP4 expression in cultured smooth muscle cells (88). This suggests that MRP4 overexpression could compensate for PDE inhibition. In cardiomyocytes, MRP4 deficiency is associated with changes in the expression profile of PDEs. PDE3A and PDE4A expression and activities were increased, perhaps as a way to compensate for the loss of cAMP extrusion (89). Counteracting PDEs may thus blunt the impact of MRP4 deficiency, and the reverse is likely true even if it has not been formerly established. With aging, the overactivity of PDEs progressively decreases, and MRP4-deficient mice display enhanced cardiac myocyte cAMP formation and an enhanced effect on cardiac contractility and remodeling (89). Clearly, more thorough investigations of PDE inhibitors, their potential to inhibit MRP4, and MRP4's potential to blunt their effect are needed to assess whether MRP4 inhibition accounts for some of the therapeutic effect of PDE inhibitors (Figure 6).

TRANSPORTERS AS REGULATORS IN DISEASE

Cardiovascular System

Cyclic nucleotides are key determinants of numerous cardiovascular functions. In the vascular system, cAMP causes vasorelaxation, inhibits smooth muscle cell proliferation, and regulates en-dothelial permeability. The underlying mechanisms involve activation of cAMP-dependent PKA and phosphorylation of PKA substrate proteins. cAMP can also act in a PKA-independent manner through its direct binding to Epac (see above). In contrast,

soluble guanylyl cyclase activation in response to nitric oxide (NO) stimulation increases the cGMP levels and causes vasodilation and inhibition of smooth muscle cell proliferation. Determinants of the NO/cGMP pathway remain incompletely described, but the antiproliferative effect of cGMP may involve inhibition of key targets through PKA and/or protein kinase G activation (90). One potential mechanism accounting for this could be increased cAMP levels, which produce inhibition of cAMP-hydrolyzing PDE3, as shown in proliferating vascular smooth muscle cells.

Reagents that can augment cyclic nucleotide levels represent good candidates to limit vascular remodeling and promote vasodilation. Targeting cyclic nucleotide degradation by inhibition of PDEs has been a therapeutic goal for many years. Members of the PDE family have specificity for cAMP or cGMP, but also differential tissue expression. In the vascular system, PDE5 represents the major metabolic pathway for cGMP, whereas PDE3/4 catalyzes cAMP degradation. PDE5 inhibitors are used in patients affected with vasculoproliferative disorders such as pulmonary hypertension.

Recent evidence has highlighted the role of transporters in regulating the levels of cyclic nucleotides in the vascular system. Two recently published studies reported that MRP4 acts as an endogenous regulator of intracellular cyclic nucleotide levels and as a mediator of related signaling pathways in vascular smooth muscle cells (72, 89). MRP4 knockdown, achieved by RNA interference (RNAi), in human arterial smooth muscle cells significantly increased cAMP and cGMP intracellular levels while concomitantly decreasing extracellular levels of the cyclic nucleotides. However, the maximal increase was observed by inhibition of both PDE and MRP4, suggesting that the intracellular cyclic nucleotide content is determined by two independent mechanisms, efflux by MRP4 and catabolism by PDEs.

Vascular remodeling under pathological conditions is influenced by MRP4 function. MRP4 is highly expressed in proliferating smooth muscle cells, but expression is low in quiescent vascular cells (72, 89, 91); this result suggests that MRP4 may play a minor physiological role in the normal vascular system. However, MRP4 expression and the contribution of MRP4 to function may increase under pathological conditions because MRP4 silencing significantly inhibits proliferation of cultured smooth muscle cells (72). In vivo, MRP4 silencing achieved by RNAi-based gene transfer has the potential to significantly reduce the formation of the pathological proliferative layer (i.e., the neointima) after injury of rat carotids (72). Interestingly, these beneficial effects were linked mainly to the activation of PKA, which amplifies the phosphorylation of CREB (cAMP-responsive element binding protein), a classical repressor of smooth muscle cell proliferation (91).

Recent studies on mice and in patient samples suggest that inhibiting MRP4 could help in the treatment of pulmonary arterial hypertension (PAH), a vasculoproliferative and vasospastic disorder involving abnormalities in the homeostasis of cyclic nucleotides and smooth muscle cell proliferation. In a hypoxia-induced mouse model of PAH, MRP4-deficient mice, unlike wild-type mice, did not develop disease, and a small-molecule MRP4 inhibitor reversed development of PAH (88).

MRP4 in Secretory Diarrhea

CFTR is expressed at the apical surfaces of secretory epithelial cells that line the lumen of the gut, where it forms a macromolecular complex with MRP4 that is mediated by PDZK1 (see ABCC4/MRP4 as a Regulator of cAMP, above). Exposure of the gut lumen to toxins secreted by colonizing pathogenic microorganisms (e.g., *Escherichia coli*, *Vibrio cholerae*) elicits excessive production of cAMP and/or cGMP. This production leads to the hyperactivation of the CFTR channel with concurrent inhibition of fluid absorption, a process mediated by Na^+/H^+ exchangers (e.g., NHE3) and the epithelial sodium channel. This dysregulation in ionic balance causes secretory diarrhea. As described above, MRP4 functions as a cAMP and/or cGMP efflux transporter to ensure compartmentalized regulation of cAMP and/or cGMP levels. This function can be accomplished by MRP4 localization in plasma membrane microdomains, suggesting that MRP4 plays a regulatory role in the pathogenic process of enterotoxin-induced secretory diarrhea. Indeed, in vivo studies have shown that inhibition of MRP4 potentiates cholera toxin–induced and CFTR-dependent fluid secretion; MRP4-deficient mice are more prone to CFTR-mediated secretory diarrhea (71). The findings have therapeutic implications for the development of pharmacologic approaches to treat disorders such as secretory diarrhea, irritable bowel syndrome, and perhaps inflammatory bowel disease.

CONCLUSIONS AND IMPLICATIONS

The general view has been that PDEs provide the sole means of modulating cAMP in cells (92, 93). However, as we discuss here, ABC transporters provide an additional means of regulating cAMP levels. These transporters can form macromolecular complexes, but whether the formation of the complex is dictated by the stimulus is unknown. Also unknown is the extent to which the overlap in activity between inhibitors of PDEs and ABC transporters provides therapeutic benefit or untoward side effects. Such issues, in addition to efforts to sort out the roles of certain less characterized ABC transporters, are challenges for the future.

Acknowledgments

This work was supported by NIH grants 2R01 GM60904, P30 CA21745, and CA21865 and by the American Lebanese Syrian Associated Charities (ALSAC).

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SUMMARY POINTS

1. PDEs can regulate cyclic nucleotide concentrations at the plasma membrane as well as in the cytosol.
2. Membrane cyclic nucleotide concentrations can be regulated by PDEs and ABC transporters.
3. Some PDE inhibitors also inhibit ABC transporters.
4. Multiple ABC transporters can transport cyclic nucleotides.
5. ABC transporters form multiprotein regulatory complexes.
6. ABC transporters modulate PKA signaling.

FUTURE ISSUES

1. Do drugs that inhibit ABC transporters contribute to their thereapuetic efficacy (e.g., antiplatelet drugs)?
2. Does the composition of the multiprotein complex have an impact on ABC-transporter activity?
3. What is the interplay between ABC transporters in regulating cyclic nucleotides?
4. How do PDEs and ABC transporters coordinately regulate membrane and cytosolic cyclic nucleotide concentrations?

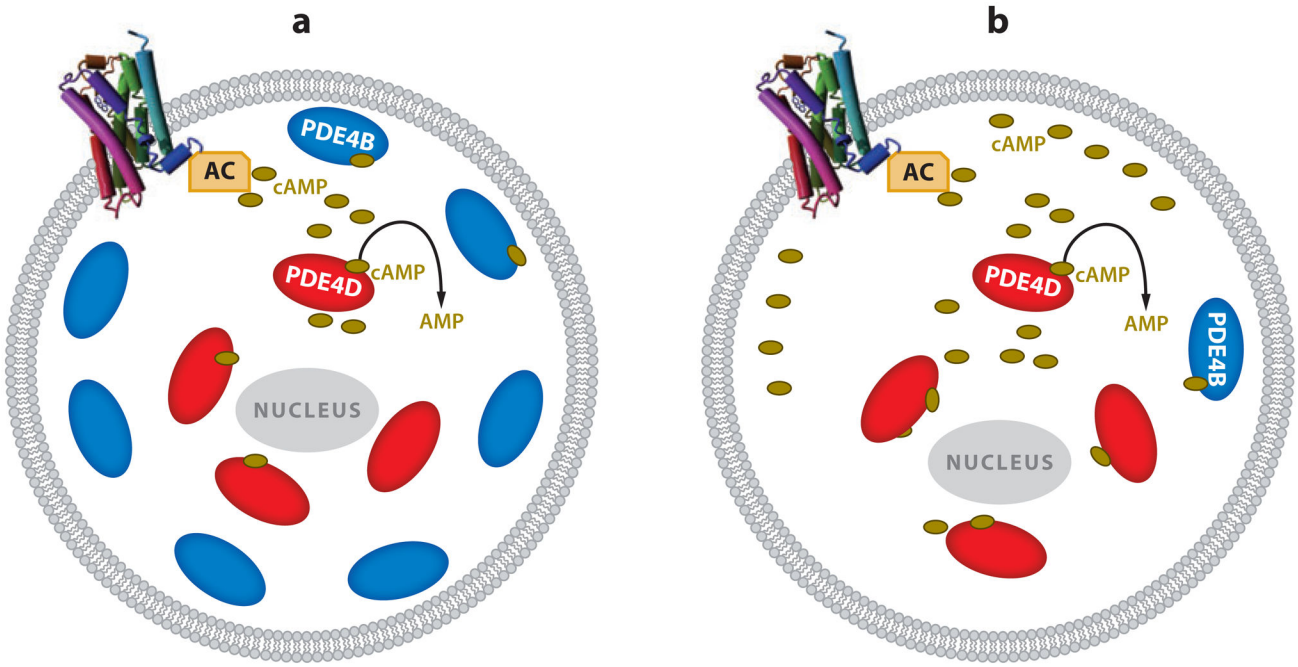

Figure 1.

Role of compartmentalized phosphodiesterases (PDEs). (*a*) In response to stimulation by an agonist, concerted activity of PDE4B at the subplasma membrane and PDE4D in the cytosol generates an intracellular cyclic adenosine monophosphate (cAMP) gradient. (*b*) cAMP concentrations in the cytosol are regulated by the compartmentalized PDE4D. Abbreviation: AC, adenylate cyclase.

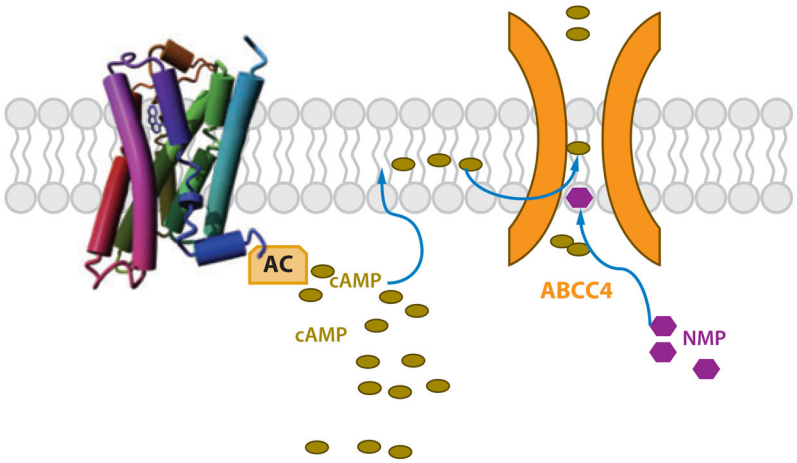

Figure 2.

ABCC4 at the plasma membrane regulates the concentrations of intracellular cyclic nucleotides and NMP analogs. Abbreviations: AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; NMP, nucleotide monophosphate.

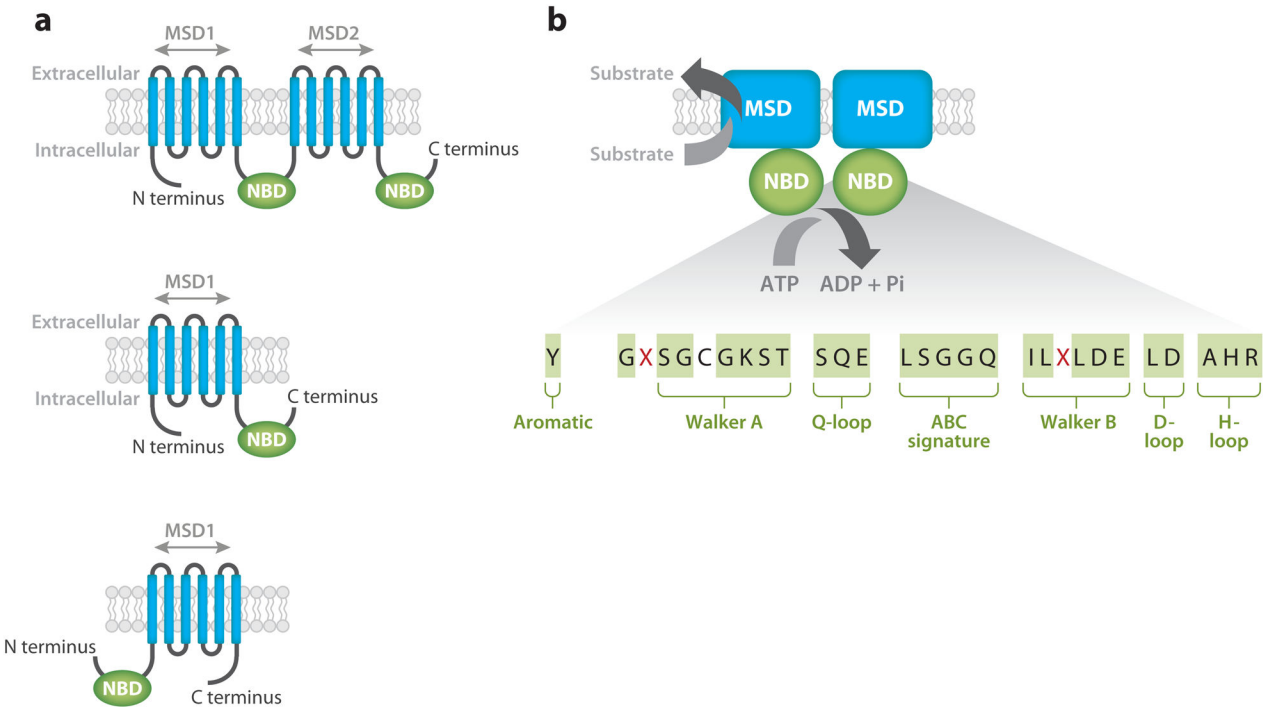

Figure 3.

(*a*) Membrane topology of full and half ATP-binding cassette (ABC) transporters. Blue bars represent predicted membrane-spanning domains (MSDs); green circles represent nucleotide-binding domains (NBDs). (*b*) The core domain structure of ABC transporters with the conserved key motifs in a NBD. Abbreviation: Pi, inorganic phosphate.

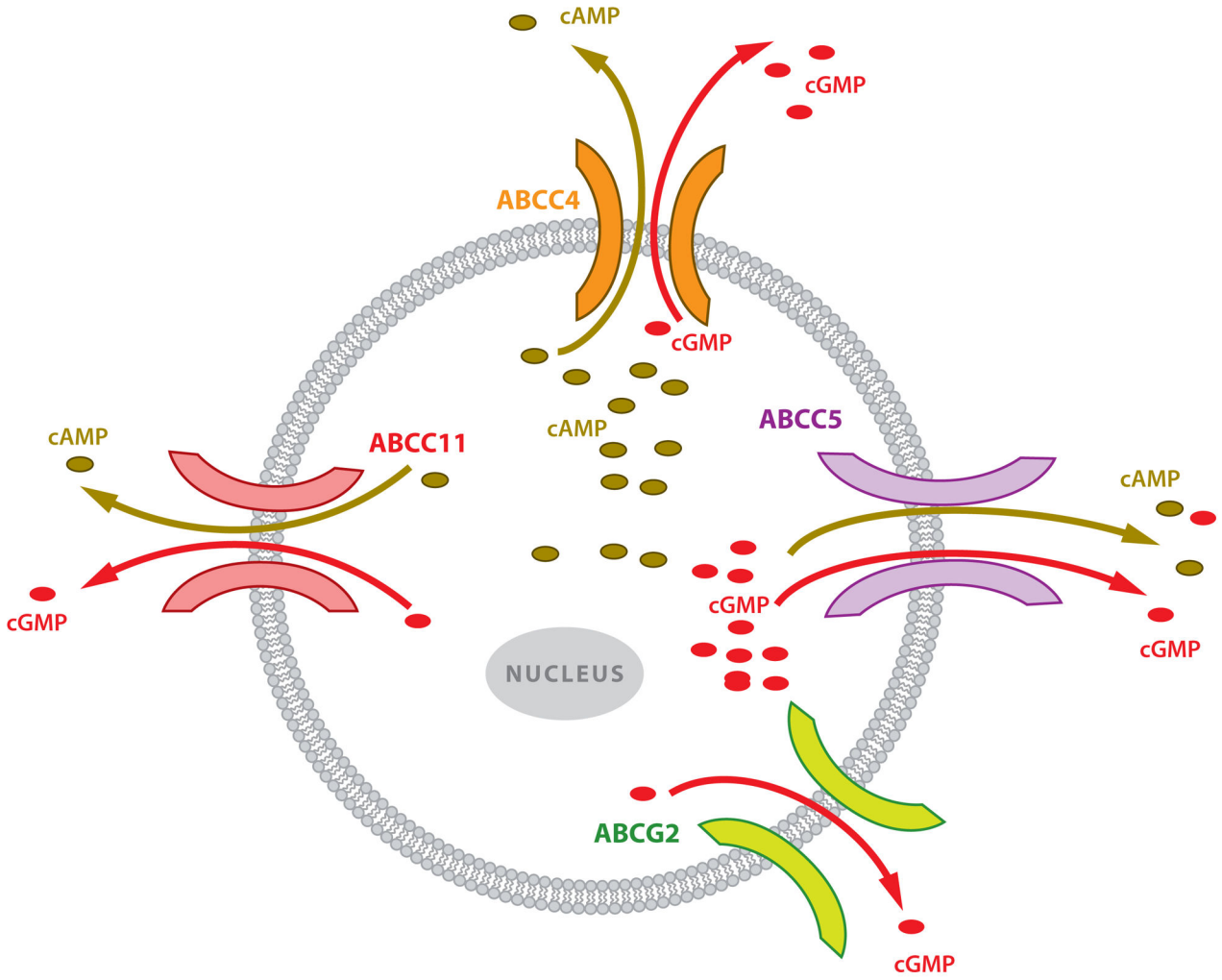

Figure 4.

Multidrug transporters export intracellular cyclic nucleotides with different specificity: ABCC4, ABCC5, and ABCC11 can transport both cAMP and cGMP, whereas ABCG2 transports only cGMP. Abbreviations: cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate.

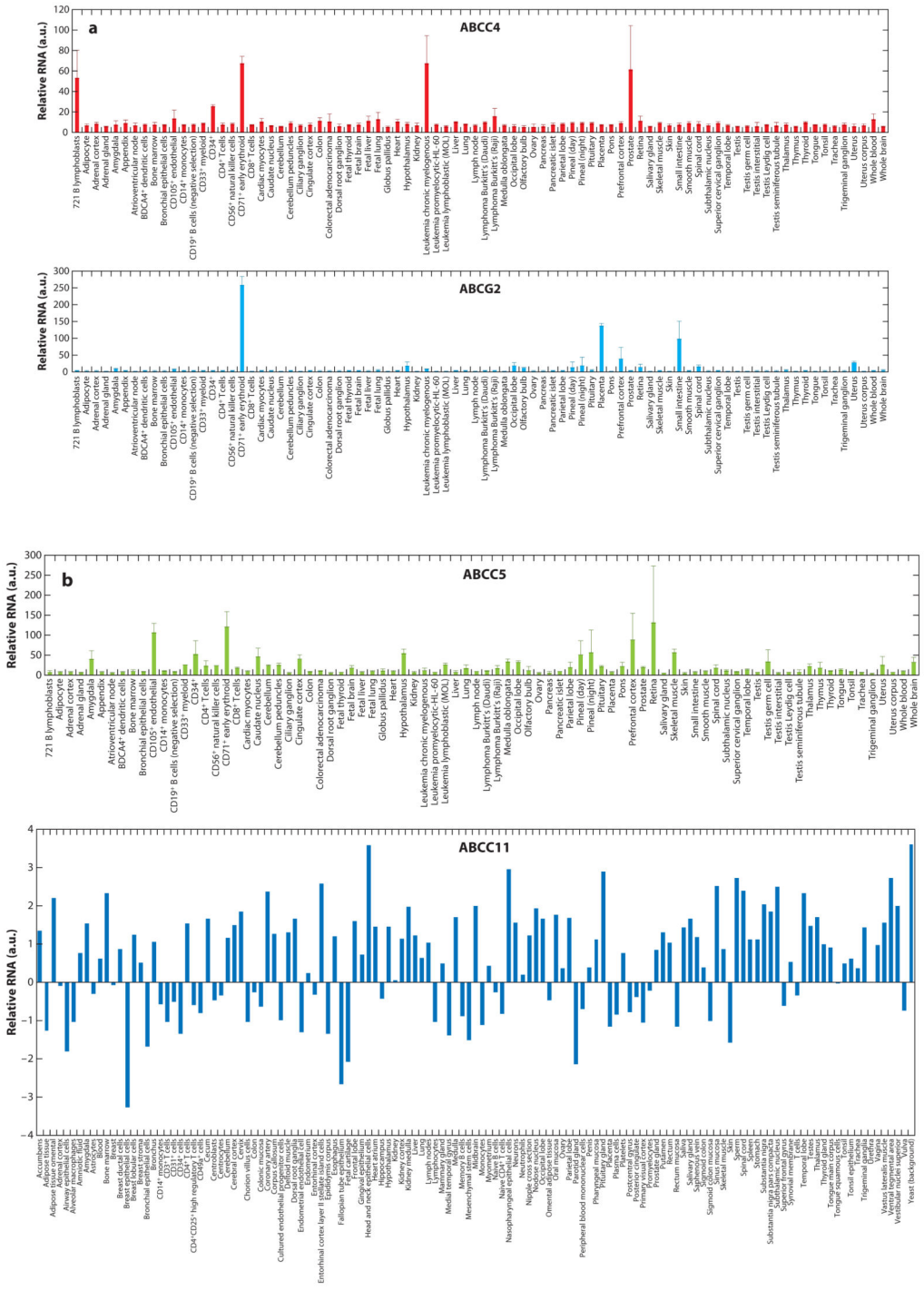

Figure 5.

Tissue expression of ATP-binding cassette (ABC) transporters that export cyclic nucleotides. Each cyclic nucleotide–transporting ABC transporter has a distinct tissue expression pattern. The overlap in tissue distribution suggests that some tissues may have redundant backup cyclic nucleotide export routes. In contrast, ABCC11 is broadly expressed

and may impact multiple tissues. Data from http://www.biogps.org. Abbreviation: a.u., arbitrary units. Figure continues on next page.

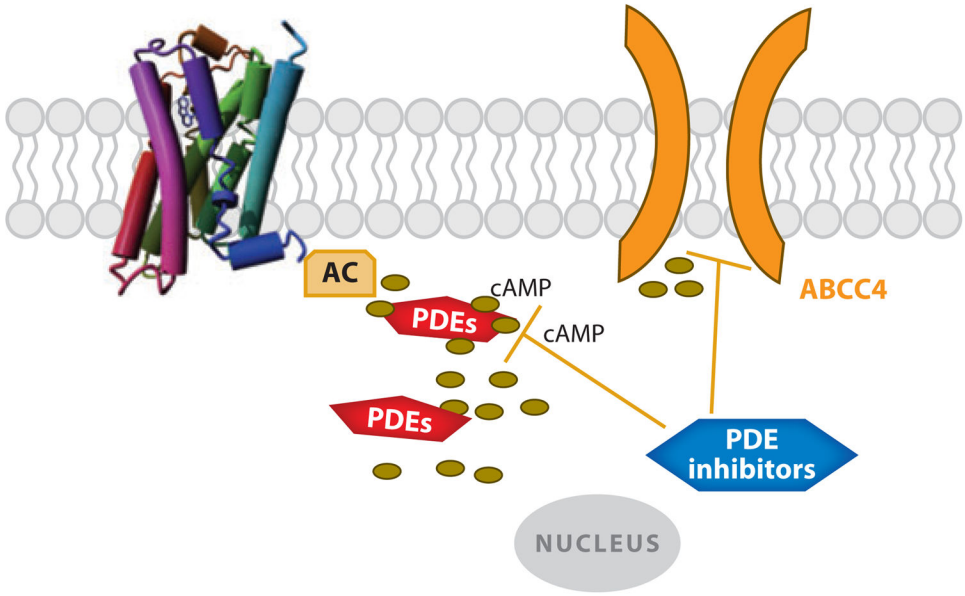

Figure 6.

Phosphodiesterase (PDE) inhibitors may dually inhibit ABCC4 and PDEs. Abbreviations: AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; PDE, phosphodiesterase.

Table 1

ABC transporters that transport cyclic nucleotides

Transporter	Species	System	Nucleotide	K_m	Reference
ABCG2	Human	Erythrocyte vesicles	cGMP	132 μM	80
Abcg2	Mouse	Erythrocyte vesicles	cGMP	9 mM	
Abcc4	Mouse	Erythrocyte vesicles	cGMP	2.9 mM	80
ABCC4	Human	SF9 insect cell vesicles	cAMP	44.5 μM	48
			cGMP	9.69 μM	
ABCC4	Human	SF9 insect cell vesicles	cAMP	Not reported	49
			cGMP		
ABCC4	Human	HEK293 cells	cAMP	Not reported	50
			cGMP		
ABCC5	Human	SF9 insect cell vesicles	cAMP	Not reported	49
			cGMP		
ABCC5	Human	HEK293 cells	cAMP	Not reported	50
			cGMP		
ABCC5	Human	V79 cells	cAMP	379 μM	74
			cGMP	2.1 μM	
ABCC11	Human	LLC-PK1 vesicles	cAMP	Not reported	106
			cGMP		

Abbreviations: cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; LLC-PK1, pig kidney epithelial cells.

Annu Rev Pharmacol Toxicol. Author manuscript; available in PMC 2015 January 22.

Table 2

Phosphodiesterase inhibitors that inhibit MRP4/ABCC4

Inhibitor	PDE	IC_{50}	Reference	MRP4 IC_{50}	Estimated human concentration[b]
Cilostazol					13.5 μM
	PDE2	0.2 μM	32		
	PDE3	45.2 μM			
Dipyridamole[a]			105	2 μM	2.9 μM
	PDE5	0.9 μM	94		
	PDE6	0.38 μM			
	PDE7	9 μM			
	PDE8	4.5 μM			
	PDE10	1 μM	95		
	PDE11	0.37 μM			
Sildenafil[a]			105	20 μM	2.6 μM
	PDE1	280 nM	96		
	PDE5	3.5 nM			
	PDE6	37 nM			
Tadalafil					
	PDE5	6.7 nM	96		
	PDE11	37 nM			
Trequinsin[a]			105	10 μM	
	PDE2	~1 μM	97		
	PDE3	0.3 nM	98		
	PDE4	230–790 nM	99		
Vardenafil					
	PDE1	180 nM	100		
	PDE2	>1,000 nM			
	PDE3	>1,000 nM			
	PDE4	>1,000 nM			

Inhibitor	PDE	IC_{50}	Reference	MRP4 IC_{50}	Estimated human concentration[b]
	PDE5	0.7 nM			
	PDE6	11 nM			
Zaprinast[a]			105	250 μM	
	PDE1	6 μM	101		
	PDE5	0.76 μM	102		
	PDE6	0.15 μM			
	PDE9	35 μM	103		
	PDE10	11–22 μM	95		
	PDE11	12 μM			
Zardaverine					
	PDE3	0.58 μM	104		
	PDE4	0.17 μM			

[a] From Reference 105.

[b] Formula for calculation of human dosage concentration:

$$\text{conc of drug in the body} = \frac{\text{dosage(mg/day)}}{\text{mol wt}} \times \frac{1}{\text{total body water (40liter)}^{*}}$$

[*] Based on assumption for average 75-kg person.

Abbreviations: ABC, ATP-binding cassette; PDE, phosphodiesterase.