CHAPTER

3

Membrane Transport

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OUTLINE

INTRODUCTION

Primary active transporters are membrane proteins that energize many of the most basic neural functions. They transduce free energy from ATP hydrolysis into electrochemical energy that is stored in the transmembrane concentration gradients of Na^+, K^+, Ca^{2+} and protons. These energy stores are employed by membrane channel proteins for signaling, and by membrane secondary transporters that selectively concentrate many other ions and molecules. *Secondary active transporters* depend on an ion gradient to transport their specific ligands uphill across membranes and subserve many neural functions such as packaging neurotransmitters into vesicles, terminating signals at synapses and transporting metabolites. *Facilitators* are membrane proteins that enable specific molecules to diffuse across membranes, often under regulatory control, but they are incapable of 'uphill' transport (Fig. 3-1).

Relative to ion channels, which upon opening allow diffusion of thousands of ions per channel per millisecond (Ch. 4), transporters typically require milliseconds to move 1–3 ions or molecules per reaction cycle in the uphill direction. Thus transporters are usually expressed at much higher densities in membranes than are ion channels.

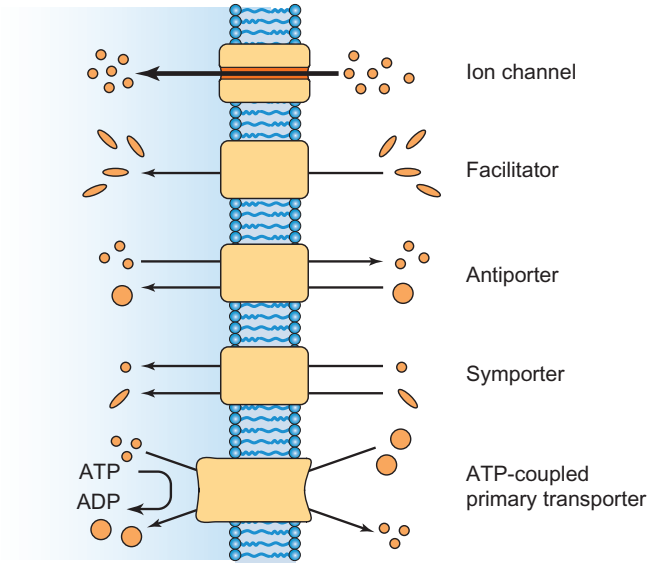

FIGURE 3-1 **Types of membrane transport proteins.** Ion channels provide gated diffusion paths across cell membranes that are regulated by membrane voltage, interactions with ligands, and/or phosphorylation. Facilitators or uncoupled transporters provide highly selective pathways, e.g., for D-glucose or water, but are not coupled to energy sources and therefore cannot concentrate their substrates. Transporters that are coupled to energy sources can alter the steady-state distribution of their substrate ions and/or molecules. Secondary transporters derive energy from existing ion gradients to transport a second ion or molecule uphill in a direction that is either the same as (symport) or opposite to (antiport) that of the energizing ion. Primary transporters couple a chemical reaction to protein conformational transitions, which supply energy to generate concentration gradients of one or more substrates across cell membranes.

PRIMARY ACTIVE TRANSPORT (P-TYPE) PUMPS

The primary transporters discussed in this chapter belong to three distinct genomic superfamilies that differ markedly in structure and reaction mechanism. These are P-type, V_0V_1-type

and ABC-type. These superfamily members all catalyze reactions with ATP that drive conformational cycles of the respective proteins to move substrates across membranes and 'uphill' to higher concentrations. *P-type transporters*, including the sodium/potassium pump, primary membrane calcium pumps and the sarcoplasmic/endoplasmic reticulum calcium pumps, share the same general reaction mechanism. Most pumps belonging to the P-type transporter superfamily have evolved to create cation gradients. In the case of the P-type Na^+, K^+ pumps, the Na^+ and K^+ concentration gradients generated across all eukaryotic plasma membranes constitute a large potential energy store that is employed for neuronal conductance, electrical signaling and driving secondary transporters. Ca^{2+} gradients are also generated by P-type transporters, but these gradients are employed primarily for regulating intracellular signaling (Ch. 24). The structures and reaction mechanisms of the Na^+, K^+ and Ca^{2+} pumps are similar and have provided insights into the functioning of the whole class of P-type transporters. Cu-ATPases function in the transport of Cu^+ into liver cells and bile and their mutated forms underlie certain neurologic diseases mentioned later. *V_0V_1 pumps* are H^+ pumps in Golgi-derived vesicles while *ABC cassettes* function in the transport of various large molecules and certain pharmacologic substances.

Na,K-ADENOSINETRIPHOSPHATASE (Na,K-ATPASE)

The reaction mechanism of Na,K-ATPase illustrates the mechanism of P-type pumps

The Na^+, K^+ pumps are driven by a cycle of conformational transitions of the protein energized by phosphorylation of their catalytic sites. Cytoplasmic ATP binds to the catalytic site and the phosphorylation is activated by binding of cytoplasmic Na^+, followed by hydrolysis of the phosphorylated site, which is activated by binding of extracellular K^+ to extracellular oriented sites (Fig. 3-2). The cycle is initiated by ATP binding to the catalytic site and cytoplasmic Na^+ binding to all three ionophoric sites. The catalytic sites contain an aspartyl residue, which becomes phosphorylated only when all three Na^+-binding sites are occupied by sodium ions. Phosphorylation of the aspartyl

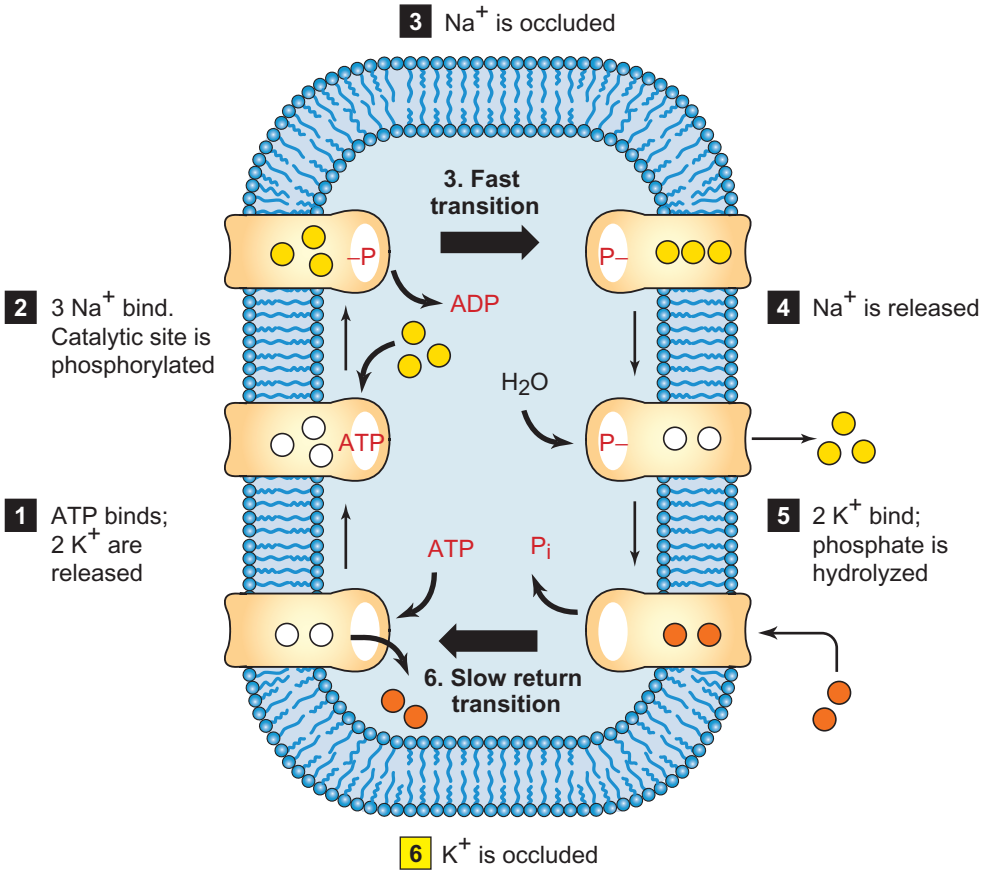

FIGURE 3-2 **The mechanism of the ATP-dependent sodium pump.** The sequence of reaction steps is indicated by the large arrows. On the left side, pump molecules are in the E1 conformation, which has high affinity for Na^+ and ATP and low affinity for K^+. Ionophoric sites are accessible only from the cytoplasmic side. Step 1. K^+ is discharged as metabolic energy is added to the system by ATP binding. Step 2. Three Na^+ bind and the enzyme is reversibly phosphorylated. Step 3. The conformational transition from E1~P to E2–P, shown at the top, is the 'power stroke' of the pump during which the ionophoric sites, with their three bound Na^+, become accessible to the extracellular side and decrease their affinity for Na^+. Part of the free energy of the enzyme acylphosphate has been dissipated in this process. Step 4. Three Na^+ dissociate from E2-P. Step 5. Two K^+ bind and more free energy is dissipated as the enzyme acylphosphate is hydrolyzed. At this point the two K^+ become tightly bound ('occluded') and in step 6 E2 reverts to E1, carrying the K^+ to the cytoplasmic side. Each step of this cycle is experimentally reversible.

is readily reversible when the pump molecule is in the E1 conformation; that is, the energy states of the E1 aspartylphosphate bond and the ATP phosphate bond are of similar high energy. This is demonstrated by the ability of the phosphorylated enzyme to rephosphorylate ADP to ATP under conditions that inhibit step 3 in Figure 3-2. However, the active pump formation of the aspartylphosphate bond initiates a rapid transition to the E2-P conformation, in which the ionophoric sites have changed their orientation and selectivity, causing the three Na^+ to be discharged extracellularly in exchange for binding two K^+. This initiates hydrolysis of the aspartylphosphate bond in the E2 state that, in concert with ATP binding at its intracellular site, causes E2 to revert to E1. In consequence of this, the ionophoric sites discharge the two K^+ into the cytoplasm. ATP and Na binding initiates the next transport cycle. This transport reaction can occur at a rate of ~10,000 cycles per minute (see (Albers, 1967) for detailed discussion of the biochemistry).

As noted above, a Na^+, K^+ pump exchanges three Na^+ for two K^+ per transport cycle. This produces a net outward flow of positive charge, which can generate an electrogenic potential. The extent of membrane hyperpolarization by the pump can be evaluated by measuring the reduction in membrane potential produced by selective Na^+, K^+ pump inhibitors such as ouabain. The electrogenic potential is usually small, 10 mV or less, because opposing ion currents flow through channels or secondary transporters in the contiguous membrane. However, in some neurons and muscle cells, sodium pump hyperpolarization can shorten the duration of an action potential and contribute to negative afterpotentials. In heart muscle, hyperpolarization due to Na^+ pumping occurs after sustained increases in firing rate. The hyperpolarization may be a factor in producing cardiac arrhythmias.

Molecular structures of the catalytic subunits in the P-type transporters are similar

The structures of the α1 catalytic subunits of Na, K-ATPase, SERCA1a and other P-type transporters contain several highly conserved domains, as illustrated for SERCA1a in Figure 3-3 (Bublitz et al., 2010). The **A** domain consists of the N-terminus and the second cytosolic domain connected to transmembrane helices M2 and M3. The enzyme also has a highly conserved phosphorylation (**P**) domain that is close to the membrane and a relatively isolated nucleotide-binding (**N**) domain. It appears that the rotation of the **A** domain opens and closes the **A, N** and **P** domains during the E1→E2 transport cycle. Both the **A** and **N** domains contain multiple functional motifs that interact with many soluble and membrane proteins. These interactions are cell specific and make it possible for the Na,K-ATPase to participate in signaling functions as well as in transport (see below).

The active Na,K-ATPase is a heterodimer consisting of a catalytic α subunit and an accessory β subunit

Four isoforms of the α subunit and three of the β subunit are expressed in mammals. The α subunits all are about 110 kDa and have about 85% sequence identity. Three of the α-subunit isoforms (α1, α 2, α 3) are expressed in brain in different cell types, to be discussed in a later section, while the α4-isoform is expressed in testis where it regulates sperm motility (Sanchez et al., 2006). In addition, a family of non-obligatory but associated γ subunits, the FXYD proteins, that modify cation affinities for the Na,K-ATPase in different tissues, are discussed later.

The alpha catalytic subunits contain the specific Na^+, K^+ and nucleotide-binding sites, the aspartyl phosphorylation site and an ouabain-binding site. The latter is exposed on the extracellular surface when the enzyme is in the E2 conformation. The cardiotonic steroids (CTS), of which ouabain is the principal example, are specific ligands and inhibitors of the enzyme hydrolytic activity (Albers et al., 1968). Ouabain binds tightly to its extracellular oriented site on the α subunit in the E2 conformation and prevents return to the E1 conformation. Ouabain binding exerts negative cooperativity with Na^+ and nucleotide binding and positive cooperativity with K^+ binding and inorganic phosphate (Pi) incorporation. The ouabain effect is likely conformational. Enzyme-bound ^{3}H-ouabain in microsomal preparations is stable to washing and filtration at neutral pH (Albers et al., 1968; Siegel & Josephson, 1972). Ouabain dissociates from the enzyme very slowly, thus maintaining the enzyme in the E2 conformation. In the ouabain–enzyme E2 conformation, the aspartyl residue cannot be phosphorylated from ATP but it does incorporate Pi, resulting in a low energy acid-stable phosphopeptide (E2-P) that cannot phosphorylate ADP, in contrast to the Na^+-dependent phosphorylation from ATP (E1~P) (Siegel et al., 1969).

The α-subunit isoforms are expressed in a cell- and tissue-specific manner

The α subunit isoforms are products of separate genes that differ in their 5′-flanking regulatory sequences (Potaman et al., 1996). Their expression is subject to a complex interplay of regulatory signals in different cells (see references in Wang et al., 2007). The α1-isoform is found in all epithelial cells and in most other cells, in which it performs what have been called "housekeeping functions." The α2- and α3- isoforms are expressed in skeletal muscle, nervous system and cardiac myocytes. In the central nervous system, Na,K-ATPase is most highly expressed in regions where high levels of Na^+/K^+ exchange are expected. These include axonal terminals, nodes of Ranvier, dendritic processes and neuronal soma, but not myelin wrappings. Alpha-3 is expressed in neurons, α1 in glia and α2 in glia and some neurons. In the rat spinal cord, neuronal α1 expression is restricted to a set of laterally situated anterior horn cells and to intermediolateral thoracic cord neurons. Rat dorsal root ganglion cells express α3 alone or together with α1 but do not express α1 alone. In retinal pigment epithelium and choroid plexus ependyma, the Na^+ pump is most concentrated on apical (luminal) surfaces (Ernst et al., 1986). With these exceptions, epithelial cells adapted for secretion or reabsorption express the Na^+ pump exclusively on the basolateral or abluminal surfaces.

The β subunits are monotopic glycoproteins and exhibit some characteristics of cell adhesion molecules

At least one of the three β-subunit isoforms must be coexpressed with the α subunit to translocate the α subunit from

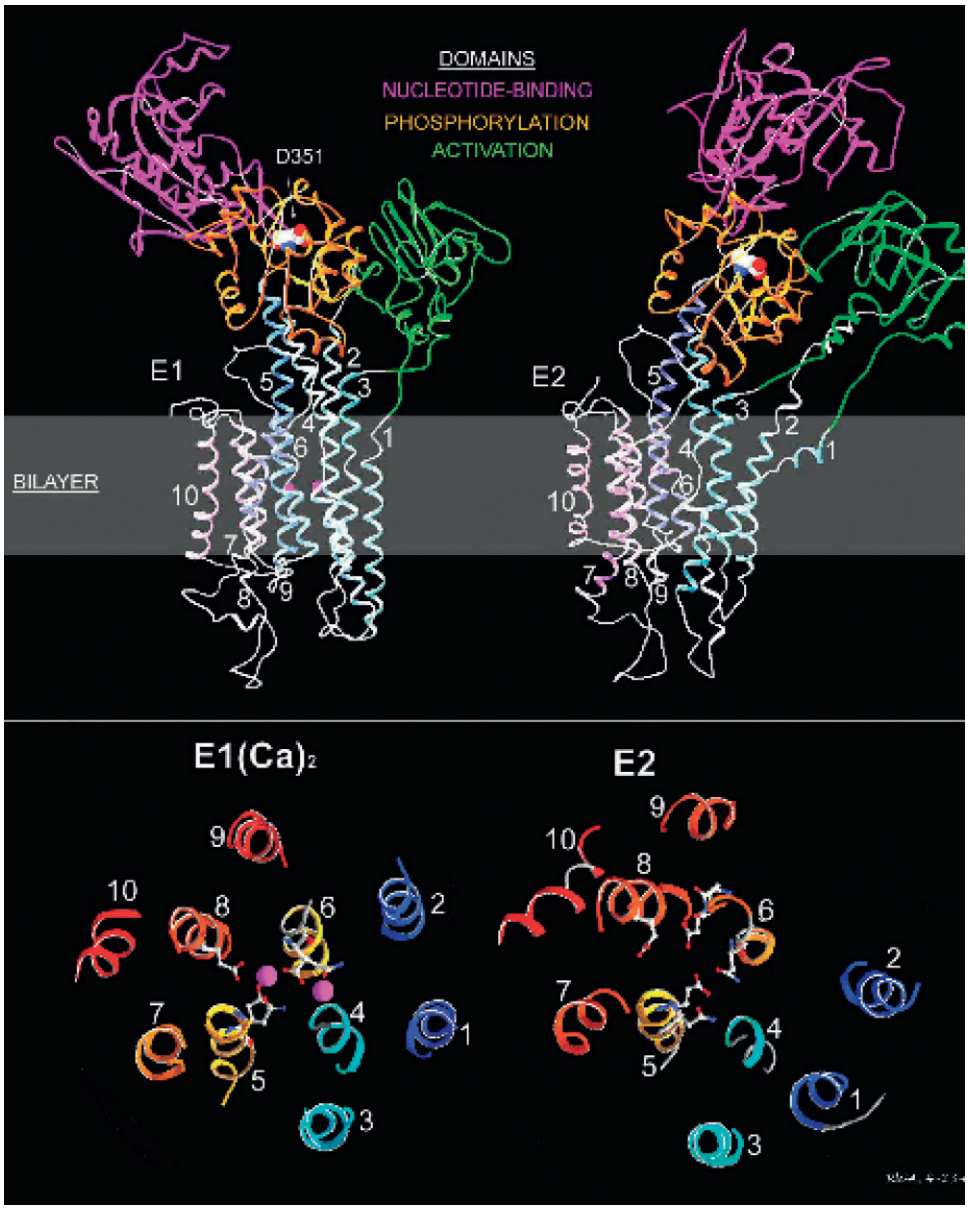

FIGURE 3-3 **Structures of the SERCA1a calcium pump.** E1$(Ca)_2$ was obtained from crystals formed in the presence of Ca^{2+}; E2 was obtained from crystals formed in the absence of Ca^{2+} and in the presence of the selective inhibitor thapsigargin (Toyoshima, et al., 2003). The two conformations are viewed in a plane perpendicular to (top) and parallel within (bottom) the lipid bilayer. The 10 transmembrane segments are numbered in their order from N- to C-terminal, and colored sequentially from blue to red. The magenta spheres represent the two Ca^{2+}-binding sites in the E1 structures and, in the lower figures, the side chains that interact with Ca^{2+} are shown in stick form for both conformations. In the top figures, the N, A and P cytoplasmic domains are colored magenta, orange and green. The aspartyl residue, D^{351}, which is phosphorylated and dephosphorylated in each pump cycle, is shown as a space-filling model. (Guex, N., Diemand, A., Peitsch, M.C. and Schwede, T., Deep View Swiss Pdb Viewer. Basel: Swiss Institute of Bioinformatics, 2001. Online at www.expasy.org/spdbv/. Models constructed from Protein Data Base coordinates 1eul and 1iwo using DeepView 3.7.)

the endoplasmic reticulum (ER) to the plasmalemma. The three β-subunit isoforms are about 40 kDa and have about 45% sequence identity. The β1 is most generally expressed (Bublitz et al., 2010). The β2 was initially described as an 'adhesion molecule on glia' (AMOG) because it is transiently expressed on the surface of cerebellar Bergmann glia during the differentiation of granule cells (Lecuona et al., 1996). The particular β subunit isoform paired with an α subunit has little effect on pump parameters. However, the different β isoforms may influence the ultimate cellular and subcellular localizations of the Na^+ pumps (Peng et al., 1997).

The Na pump has associated γ subunits

These are within a family of ~15 kDa single-span membrane proteins that are associated with sodium pump α

subunits in a cell- and tissue-specific manner. These proteins modify Na^+ and K^+ affinities but are not necessary for pump activity, nor are they expressed in all cells. The γ subunits are classed as members of the FXYD gene family and several are expressed in brain. The kidney expresses almost exclusively Na^+,K^+-pumps with α1 subunits throughout the functionally diverse segments of the nephron, but different γ subunits with differing regulatory effects are expressed in different segments. From this it is inferred that different γ subunits optimize the sodium pump for operation under the varying ionic environments along the nephron. The γ subunits regulate kinetic properties of the sodium pump specifically appropriate for different functions such as muscle contractility, neuronal excitability and solute reabsorption in nephrons (Bibert et al., 2008).

A major fraction of cerebral energy production is consumed by the Na,K pump

Most of this is required to compensate for extrusion of intracellular Na^+ that enters through channels during electrical activity and through Na^+-dependent secondary transporters during neurotransmitter recovery. Assuming typical values for Na^+ and K^+ concentration gradients, that is, $[Na^+]_e/[Na^+]_i = 12$ and $[K^+]_i/[K^+]_e = 50$, then ΔG is about 3.8 kcal (16 kJ) per mole of Na^+ exchanged for K^+. Hydrolysis of a high-energy phosphate bond of ATP may yield as much as 12 kcal/mol under physiological conditions, thus permitting the exchange of about three equivalents of cation for each mole of ATP hydrolyzed. From this stoichiometry, it appears that the chemical free energy of ATP hydrolysis must be utilized with about 95% efficiency ($[3 \times 3.8/12] \times 100$) to operate the sodium pump under physiological conditions. Other processes utilizing energy stores are listed in Table 11-1 (Ch. 11).

Na,K-ATPase Expression patterns change with development, aging and dementia

The Na,K-ATPase activity, relative to total brain protein, increases about tenfold just prior to the stage of rapid myelination in rats during 2–12 days postnatally, corresponding to the time of glial proliferation, elaboration of neuronal and glial processes and increasing neuronal excitability (Knapp et al., 2000). Developmental changes in Na,K-ATPase in brain appear to depend on thyroidal influences. Glial cell cultures from one-day-old rat brain express progressively increasing amounts of α1-, α2-, β1- and β2-isoforms as a function of increasing triiodothyronine exposure (Banerjee & Chaudhury, 2001).

In cerebral cortex of human and rat, α3-isoform mRNA is found clustered over pyramidal and other neuronal soma while α1-isoform mRNA is distributed diffusely through the neuropil in astrocytes. In a non-demented, aged human (78 y/o) the expression of α1-isoform mRNA is not significantly changed compared to that in a young adult (39 y/o), although there are significant but small reductions in α3-isoform mRNA of neuronal perikarya. In contrast, in an individual with dementing Alzheimer's disease (78 y/o), the content of α3-isoform mRNA in neuronal perikarya is markedly diminished during the neurodegenerative process (Chauhan et al., 1997).

Na,K pump content in plasmalemma is regulated by its rapid endocytic–exocytic cycling

The regulation is demonstrated in renal proximal tubule epithelia that secrete dopamine (DA), which acts on autocrine D1-like receptors to remove Na,K pumps from their basolateral plasma membrane by endocytosis (Fig. 3-4). When liganded with DA, the G protein-coupled D1-like receptors initiate activation of PLA2 whose catalytic products are arachidonic acid and the eicosanoid 20-HETE, which together activate PKC. Activated PKC phosphorylates the Ser18 amino acid on the α subunit of Na,K-ATPase and leads to translocation and activation of PI3-K. The latter and Ser18 phosphorylation are required for binding of activator protein 2 (AP-2) to the Tyr537 site on the α subunit and subsequent recruitment of clathrin to bind to the α subunit (Doné et al., 2002). Inhibitors of PLA2 or of PKC inhibit both the activation of PI3-K and endocytosis. The stimulatory action of arachidonic acid and 20-HETE on PI3-K is abolished by inhibition of PKC. The Na,K-ATPase subunits are enclosed in clathrin-coated vesicles (CCV) and are transferred through the early and late endosomes for degradation and recycling. In contrast, bromocriptine, a powerful agonist at renal D2-like receptors, leads to increases in Na,K-ATPase content in renal proximal tubules through recruitment of increased numbers of tyrosine-phosphorylated α1 subunits.

Parathyroid hormone (PTH) can inhibit Na,K-ATPase by 50% in renal proximal tubules. PTH-receptor binding stimulates Ca-dependent ERK phosphorylation via Src kinase and PLC. ERK activation stimulates phosphorylation and activation of PLA2. The latter's catalytic products activate and translocate PKCα, leading to its phosphorylation of the α subunit and the latter's endocytosis. The PTH path differs from the inhibitory DA path in going through activation of Src kinase, PLC and Ca-dependent ERK phosphorylation (Khundmiri et al., 2008).

Recruitment of Na^+,K^+ pumps from the endosome membrane in response to serotonin- or angiotensin-activated GPCRs requires phosphorylation of the α subunit at both Ser11 and Ser18 residues and binding of AP-1 to the α-subunit, mediated by PKCβ. In brain and kidney cells, D1 and D2 receptors can each be found complexed with Na,K-ATPase and the DA receptors display reciprocally negative regulatory effects (Hazelwood et al., 2008). Ouabain-induced endocytosis of Na,K-ATPase is discussed later.

The distributions of α-subunit isoforms provide clues to their different physiological functions

While having 85% identity, the most substantial sequence differences among the α-isoforms occur in their N-terminal regions and in an 11-residue sequence of the large cytoplasmic loop. When assayed in cell cultures, the isoforms differ in their apparent affinities for intracellular Na^+ ($\alpha1 < \alpha2 < \alpha3$) (Arystarkhova et al., 1999) and extracellular K^+ ($\alpha3 < \alpha2 = \alpha1$) (Munzer et al., 1994). The α2-, α3- and α4-isoforms are unique conformers that specify their cellular and subcellular localization. Mutations in human α2- and α3-isoforms have been identified in separate

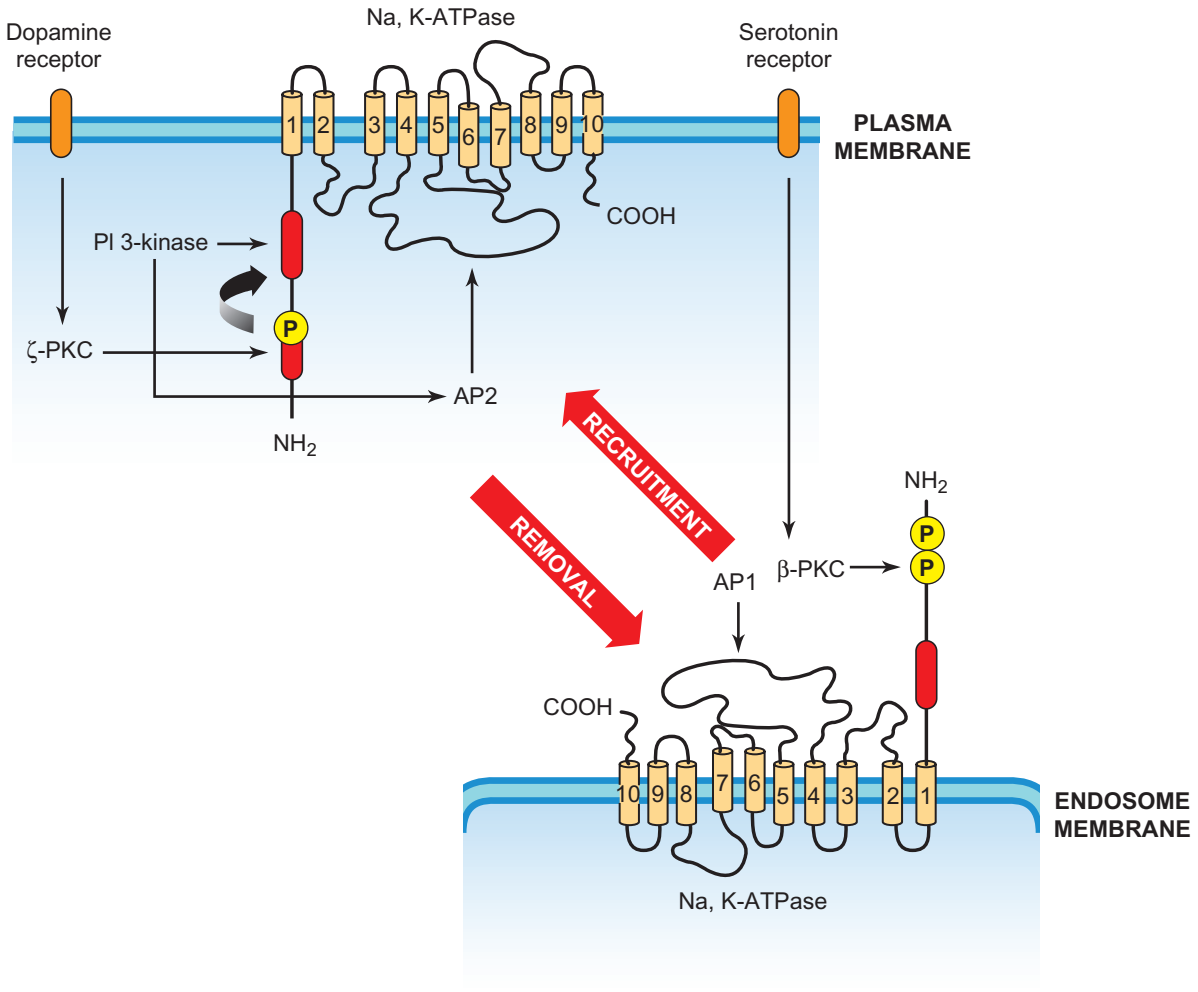

FIGURE 3-4 **Dopamine regulation of Na,K-ATPase in kidney epithelial cells that express only the α1 isoform.** Downregulation of Na, K-pumps can be initiated by dopamine via GPCR activation of endocytosis, which is controlled by phosphorylation of a single Ser-residue within the N-termini of the Na,K-ATPase α1 subunits to be internalized. Mutation analysis suggests that PI3K-IA activation ensues from its binding to a proline-rich domain present in the Na,K-ATPase α1 subunit, which only becomes accessible to the kinase after ζ-PKC-dependent Ser-phosphorylation. PI3K-IA activation recruits the AP-2 adaptors that transfer plasma membrane vesicles into early endosomes. Thus, the Na,K-ATPase serves as its own scaffold, organizing the receptor signals that ultimately downregulate its activity. Serotonin, acting through GPCR, can activate β-PKC phosphorylation of two serines within the N-termini of the endocytosed Na,K-ATPase α1 subunits. This evidently recruits AP-1 adaptors that initiate reincorporation of Na,K-pumps into plasma membrane. (Adapted with permission from Fig. 4 of Yudowsky, et al., 2000).

families with two inherited diseases: familial hemiplegic migraine type 2 for α2 (de Vries et al., 2009) and rapid onset dystonia with Parkinsonism for α3 (Brashear et al., 2007).

Regulatory factors direct the trafficking of Na,K-ATPase during its synthesis

These factors, although not understood, are responsible for directing the particular enzyme units to specific locations in the plasmalemma where their functions are coordinated with those of other membrane structures. Examples of coordination are with AMPA receptors (Zhang et al., 2009) (see Ch. 17), Ca-transporters (see later), astrocytic aquaporin 4 and glutamate transporters (see Aquaporins later) and molecules involved in signal transduction (see next).

The Na,K-ATPase/Src complex functions as a signal receptor for cardiotonic steroids (CTS)

CTS, including ouabain and marinobufagenin, are found in the systemic circulation of mammalian species. Endogenous CTS are synthesized in the adrenal cortex and brain and have been found in the hypothalamus of Milan hypertensive rats, even though the detailed biosynthesis is still poorly understood (Lingrel, 2010). Agrin, an endogenous regulator of brain function, was originally discovered as an endogenous antagonist of Na,K-ATPase α3. Through its inhibition of Na,K-ATPase α3 subunit, agrin plays important roles in Ca^{2+} homeostasis and neuronal activity (Hilgenberg et al., 2006).

Binding of endogenous CTS at sub-nanomolar ranges may not cause significant inhibition of Na,K-ATPase pumping

activity, but may provoke multiple protein kinase signaling events including ERK cascades, PLC/PKC pathways, PI3K/Akt signaling and mitochondrial production of reactive oxygen species (ROS) (Fig. 3-5) (Li & Xie, 2009). As described in Figure 3-5, Na,K-ATPase directly interacts with Src, a non-receptor tyrosine kinase, forming a receptor complex for CTS to relay its extracellular binding to intracellular signaling events. At least two pairs of domain–domain interactions are involved. The Na,K-ATPase second cytosolic domain binds Src SH2 domain, while the N domain directly associates with Src kinase domain and inhibits Src activation. The Na,K-ATPase conformational changes induced by the CTS binding provide the driving force to release the Src kinase domain from the N domain, and then to trigger the activation of Src, which transactivates receptor tyrosines such as EGF receptor, resulting in the assembly and activation of protein kinase cascades such as Ras/Raf/ERK and PLC. The activation of PLC generates IP_3 and diacylglycerol, leading to increases in cytosolic Ca^{2+} and the activation of PKC. Thus, the Na,K-ATPase can form a Src-coupled receptor. While the Na,K-ATPase provides the ligand-binding site, the associated Src acts as a signal transducer, capable of converting and amplifying the binding signal through lipid and protein kinase cascades (see Li & Xie, 2009 for references). While all the ouabain effects on Na,K-ATPase may be conformational, it is not known whether the CTS *signaling* site on Na,K-ATPase is the same as the extracellular *ouabain-binding* site that produces the enzyme inhibition and ouabain-induced incorporation of Pi into microsomal Na,K-ATPase that has been observed (Siegel et al., 1969; Siegel & Josephson, 1972).

Na,K-ATPase undergoes endocytosis upon activation of Na,K-ATPase signaling by CTS (see Fig. 3-5). Inhibition of Src or PI3K prevents CTS-induced endocytosis of Na,K-ATPase, suggesting their involvement. Functionally, CTS-induced endocytosis of Na,K-ATPase serves as a basis to terminate the signal, to relay the signal or to target it to specific intracellular compartments. It is also involved in the regulation of other

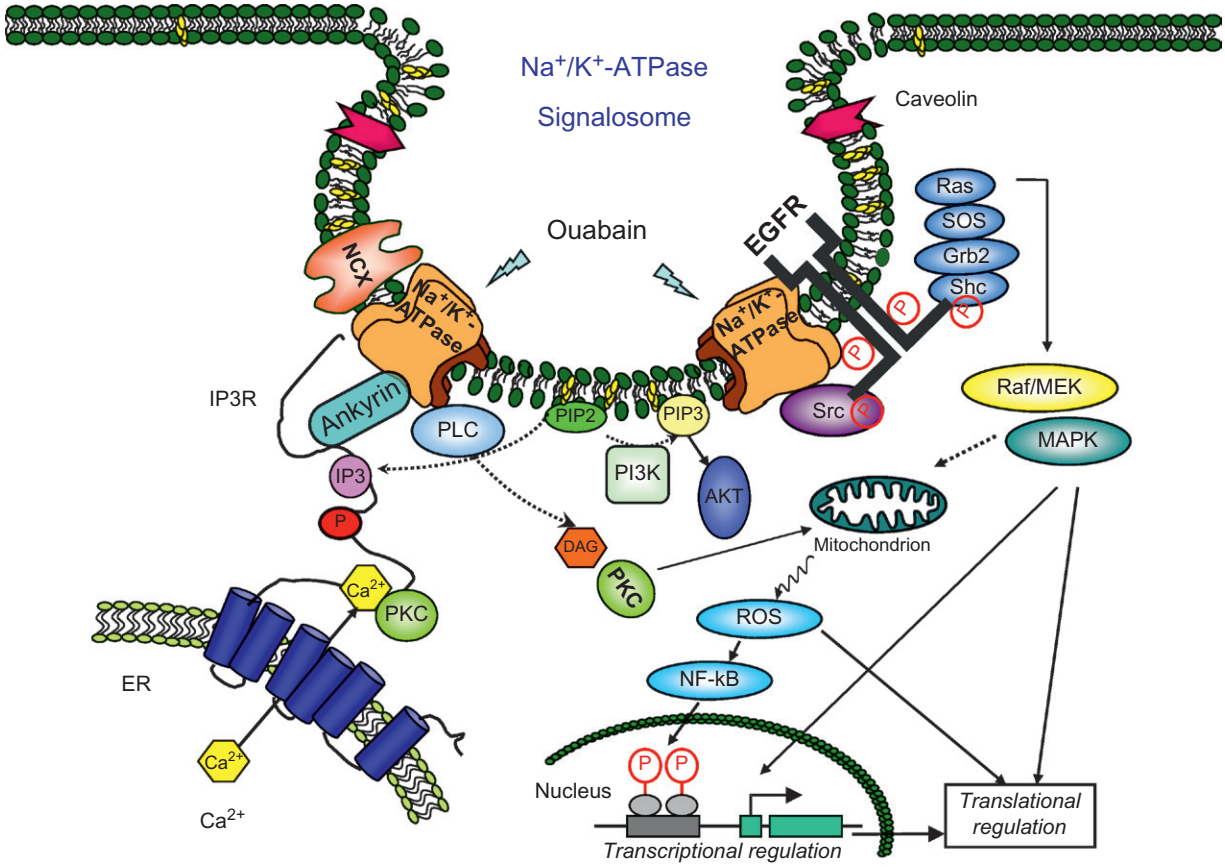

FIGURE 3-5 **Schematic presentation of Na,K-ATPase-mediated signal transduction.** Na,K-ATPase forms a signaling complex composed of multiple structural proteins (ankyrin, caveolin), receptors (IP3R, EGFR), and protein and lipid kinases (Src-kinase, PI3K). Ouabain binding to an extracellular site on Na,K-ATPase induces conformational changes in the enzyme that alter its interactions with the intracellular proteins. Ouabain binding leads to activation of both the Src/EGFR/Ras/Raf/MEK/ERK kinase cascade and the PI3K/Akt pathway. These events in turn promote PLC-catalyzed production of IP_3 and DAG, which activate IP_3R in the ER membrane and PKC, respectively. IP_3R, depicted as the 6-TM structure in the ER membrane, is the Ca^{2+} channel that will release Ca^{2+} (**yellow hexagon**) from the ER to the cytoplasm in response to an increase in IP_3. Ankyrin is involved in organizing the Na,K-ATPase –IP_3R complex. Akt, protein kinase B; DAG, diacylglycerol; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ERK, extracellular-signal regulated protein kinase; IP3, inositol 1,4,5-trisphosphate; IP3R, IP3 receptor; NCX, Na,Ca antiporter; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PKC, protein kinase C; PLC, phospholipase C; ROS, reactive oxygen species. The large red arrows depict caveolin in the flask-shaped caveolae. See also Figure 3-6 for participation of P-type pumps and mitochondria in overall Ca^{2+} homeostasis.

membrane transporters, such as the coordinated down-regulation of basolateral Na,K-ATPase and apical Na/H exchanger (NHE3) trafficking in proximal tubular cells so that the pump and leaks can be matched (Cai et al., 2008a). For detailed references and reviews of the physiological and pathological functions of CTS, please see Lingrel, 2010; Li & Xie, 2009.

Domain-specific interactions make the Na,K-ATPase an important scaffold in forming signaling microdomains

In addition to binding Src, the Na,K-ATPase interacts with many other proteins. For example, the third cytosolic domain (CD3) of Na,K-ATPase interacts with moesin, which links to actin and modifies ankyrin binding (Kraemer et al., 2003); PLC-γ (Li & Xie, 2009); adaptor protein 2 (Doné et al., 2002); cofilin, an actin-modulating protein (Kim et al., 2002); and arrestin 2 and spinophilin, which modulate GPCRs and Na,K-ATPase endocytosis (Kimura et al., 2007). The proline-rich motif within the N-terminus of Na,K-ATPase is accessible to the SH3 domain of PI3K p85 subunit and this interaction also regulates endocytosis of Na,K-ATPase (Yudowski et al., 2000). The N-terminus also interacts with IP_3R, NCX and caveolin-1. These interactions bring the transporters and their regulatory proteins together to form cell-specific signaling complexes that allow spatial and temporal regulation of signal transduction and coordination with transmembrane transport. Moreover, these interactions are important for establishing stable membrane structures such as lipid rafts (Cai et al., 2008b) (see Ch. 2). Coordinated oligomolecular complexes of Na,K-ATPase with calcium transporters, glutamate transporters, aquaporin 4, Kir4.1 channels and mGluR5 in astrocyte perisynaptic membranes, and AMPA receptors are discussed in the relevant sections below.

Unexpected roles of the Na,K-ATPase through its scaffolding partners are also indicated in regulation of caveolae, cell motility and tight junctions (Barwe et al., 2005; Cai et al., 2008b; Rajasekaran & Rajasekaran, 2009). Bindings between PI3K and Na,K-ATPase α1 subunit, and those between annexin II and Na,K-ATPase β–subunit, regulate cell motility through the assembly of Na,K-ATPase/annexin II/PI3K complex at plasma membranes (Barwe et al., 2005). The Na,K-ATPase also associates with PP2A and ouabain reduces PP2A activity, induces phosphorylation of occludin, and therefore modulates tight junction functions in epithelial cells (Rajasekaran & Rajasekaran, 2009). Interestingly, the Na,K-ATPase β-subunit in glial cells also mediates neural-astrocyte interactions (Lecuona et al., 1996).

Ca ADENOSINETRIPHOSPHATASES AND Na,Ca ANTIPORTERS

ATP-dependent Ca^{2+} pumps and Na,Ca antiporters act in concert to maintain a low concentration of free cytosolic Ca^{2+}. The concentration of cytosolic free calcium ion, $[Ca^{2+}]_i$, in unstimulated cells is between 10^{-8} and 10^{-7} mol/l, which is more than 10,000-fold lower than extracellular free Ca^{2+}. Most intracellular Ca^{2+} is stored in the endoplasmic reticulum. Because cytoplasmic $[Ca^{2+}]$ has many different intracellular signaling functions, its regulation is complex and its impairment can be catastrophic (Fig. 3-6). The coordinated regulation of cytosolic $[Ca^{2+}]$ by Ca pumps, Na, Ca antiporter and Na,K-ATPase is discussed below.

THE PRIMARY PLASMA MEMBRANE Ca TRANSPORTER (PMCA)

PMCA is a plasmalemma P-type pump with high affinity for Ca^{2+}

The K_m = 100–200 nmol/l for Ca^{2+} but the transport capacity is relatively low. The stoichiometry of PMCA is one Ca^{2+} transported for each ATP hydrolyzed. These pumps probably do not carry out bulk movements of Ca^{2+} but, having a high affinity, are most effective in maintaining very low concentrations of cytosolic Ca^{2+} in resting cells. A distinguishing characteristic of the PMCAs is that, in addition to binding Ca^{2+} as a substrate, they require activation at very low Ca^{2+} levels by binding calmodulin, the effect of which is to increase the affinity of the substrate Ca^{2+} site by 20- to 30-fold. This highly cooperative activation mechanism makes the PMCAs very sensitive to small changes in cytosolic $[Ca^{2+}]_i$. At least four PMCA genes (ATP2B1–4) form a multigene family but alternative splicing leads to many more isoforms, which are differently expressed and regulated in different cell types. The isoforms are activated also by acid phospholipids, protein kinases and other factors. Three isoforms, PMCA1–3, occur in brain; each has a distinct distribution (Di Leva et al., 2008).

SMOOTH ENDOPLASMIC RETICULUM CALCIUM PUMPS (SERCA)

SERCA, another P-type Ca pump, was first identified in sarcoplasmic reticulum

There are three isoforms of SERCA that are products of separate genes. SERCA-1 is expressed in fast-twitch skeletal muscle; SERCA-2a in cardiac/slow-twitch muscle; SERCA-2b, an alternatively spliced form, is expressed in smooth muscle and non-muscle tissues; and SERCA-3 is expressed in endothelial, epithelial, and lymphocytic cells and platelets. SERCA-2b is the major form expressed in brain, where it is found predominantly in neurons. SERCA pumps Ca^{2+} from cytosol into the ER for storage. Ca^{2+} is released from the ER through the IP_3 receptor (IP_3R) when it binds the signal molecule IP_3. The Ca pumps of ER normally reduce cytoplasmic $[Ca^{2+}]$ to <1 mM. However, rapid restoration of such low $[Ca^{2+}]$ subsequent to plasma membrane depolarizations requires coordinate activity of the plasmalemma Na,Ca antiporter (see discussion below).

High-resolution structural data exist for the SERCA1a Ca pump

The data illuminate the structure of all P-type transporters (Fig. 3-3). Unlike that of the Na,K pump, the catalytic subunit

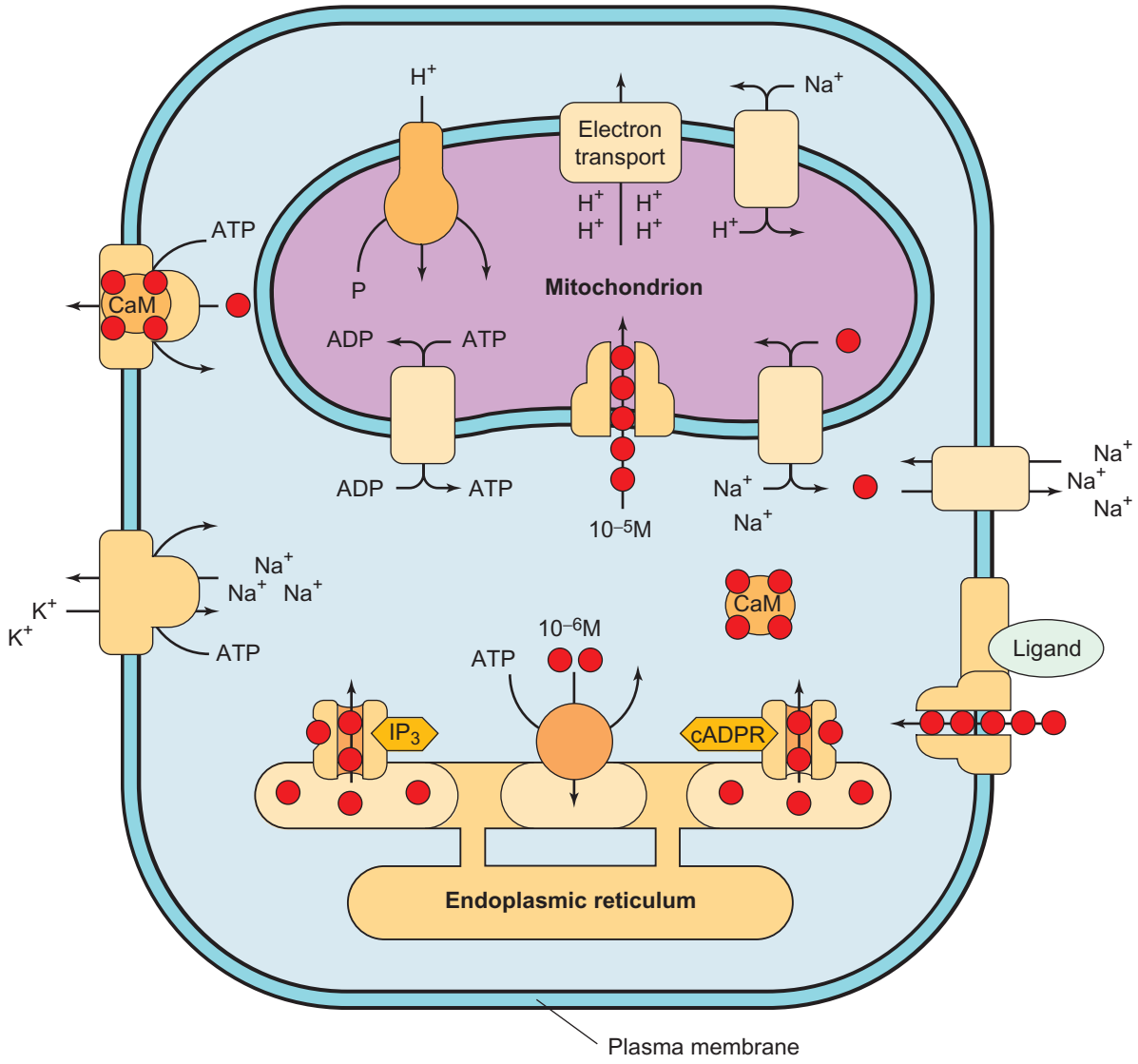

FIGURE 3-6 Calcium homeostasis. In the figure, calcium ions are represented by filled red circles. Ca^{2+} enters cells through a variety of ligand- and voltage-regulated channels, but basal cytoplasmic free Ca^{2+} is maintained at less than micromolar levels. Cytoplasmic Ca^{2+} is regulated coordinately by a Na^+/Ca^{2+} antiporter in plasma membranes and by P-type Ca-ATPases in plasma membranes and endoplasmic reticulum. The driving force for Na^+/Ca^{2+} antiporter exchange is the inwardly directed Na^+ gradient, which is maintained by $\alpha 2$ or $\alpha 3$ Na,K-ATPases. Mitochondria can participate transiently in Ca^{2+} homeostasis if the capacities of these other systems are exceeded. ER stores of Ca^{2+} can be released by second messengers, such as IP_3 or Ca^{2+} itself, in response to various receptor systems. See Figure 3-5 for proposed model of ouabain signaling and regulation of the ER stores.

of the SERCA Ca^{2+} pumps is active and does not require association with another subunit. However, the cardiac isoform, SERCA-2a, associates with a small membrane protein, phospholamban, that can regulate heartbeat strength and rate. The Ca^{2+} pump reaction mechanism is essentially the same as that illustrated in Figure 3-2 for Na,K pumps except that two Ca^{2+} are exchanged in each cycle, probably for four protons. The primary sequences for the α subunits of the Na,K- and H,K-ATPases can be superimposed on these Ca^{2+}-pump structures with only minor adjustments necessary to allow for small deletions and insertions.

Notice, in Figure 3-3, that the substrate cations are bound to ionophoric sites consisting of side chains contributed by four adjacent transmembrane helices, and that the E2 configurations of these helices are rotated relative to the E1 configurations. Examination of the paths of these helices through the bilayer does not reveal any obvious 'channel' through the membrane. This is consistent with studies of the reaction mechanism, which have shown that, at successive stages of the cycle, the cations either have access from only one side of the membrane or are 'occluded' within the pump molecule. Notice also that transmembrane helix 5, which contributes to the ionophoric domain, extends as a helix well into the cytoplasmic P domain, transforming into a β-sheet structure just at its boundary with the 'signature sequence', ICSDKTGTL. This is conserved in all P pumps and includes the aspartyl residue

(space-filled residue in Fig. 3-3), which reacts with ATP and water within each catalytic cycle.

The sodium and calcium ion pumps can be isolated to near purity and still exhibit most of the biochemical properties of the 'native' pump. Some kinetic properties of both these pumps in 'native' membranes are altered or disrupted as membrane preparations are purified. For example, when measured in intact membranes, the time dependencies of phosphorylation and dephosphorylation of the pump catalytic sites exhibit biphasic fast-to-slow rate transitions; this characteristic progressively disappears as the membranes are treated with mild detergents. One suggested explanation is that, as the pumps begin to cycle within intact membranes, the catalytic subunits associate into higher oligomers that may permit more efficient transfer of the energy from ATP into the ion transport process. There is structural evidence indicating that Na,K pumps exist in cell membranes as multimers of $(\alpha\beta)_2$.

OTHER P-TYPE TRANSPORTERS

P-type copper transporters are important for neural function

Wilson's and Menke's diseases have major neurological components (Ch. 49). The Wilson's disease–mutated gene codes for a transporter, expressed chiefly in liver, which functions in Cu^{2+} transport, and the deficit in Cu^{2+} transport leads to decreased synthesis of the Cu^{2+}-containing protein ceruloplasmin. The Menke's disease-mutated gene codes for a closely related transporter that regulates intestinal Cu^{2+} absorption (Lutsenko et al., 2007).

V_0V_1 PROTON PUMPS

The V_0V_1-ATPase pumps protons into golgi-derived organelles

V_0 is the transmembrane domain and V_1 is the cytosolic-facing domain. Their specialized neuronal function is to generate the proton–electrochemical gradient that energizes the H^+-antiporters that, in turn, concentrate neurotransmitters from the cytosol into presynaptic vesicles (Ch. 12). The V_0V_1 structure of these pumps (Fig. 3-7) is similar to that of the F_0F_1-ATP synthases and they pump protons via a similar rotor and stator mechanism. Unlike F_0F_1, which animals express only in mitochondria, the particular Golgi organelle that V_0V_1 targets depends on the isoform of α subunit that is expressed (Morel, 2003).

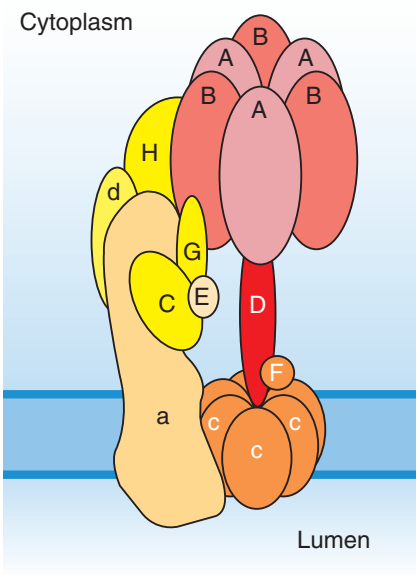

FIGURE 3-7 **Structural organization of the vesicular proton pump, V-ATPase.** V-ATPase is composed of numerous subunits organized as illustrated by color coding: the cytoplasmic domain, V1, contains eight different subunits (A–H), with three copies of the ATP binding subunits A and B; the membrane domain, termed V_0, is a hexameric ring composed of proteolipid C subunits (5 C and 1 C″) and single copies of subunits A and D. Functionally, V-ATPase consists of a rotor (white lettering) and a stator domain (black lettering). The rotor consists of the hexameric proteolipid C ring and a stalk made of subunits D and F; the stator consists of the remaining subunits, which are fixed to the membrane via subunit A. Expression of different subunit-A isoforms targets the V-ATPase to different membranes. ATP binding to and hydrolysis by the three subunits A presumably act on subunit D to induce rotation via asymmetric conformational transitions as has been described for the F_0F_1 ATP synthase. Rotation of the hexameric C ring translocates protons from the cytoplasmic interface of the proteolipid hexamer into the lumen of synaptic or Golgi-derived vesicles. The V_1 domain reversibly dissociates, in physiological conditions, to regulate V-ATPase activity. (With permission, from reference (Morel, 2003).)

ATP-BINDING CASSETTES

The ABC transporters are products of one of the largest known gene superfamilies

Of 48 ABC genes expressed in humans, evidence exists for 13 ABC transporters having some activity in brain, mainly in brain lipid transport (reviewed in detail in Kim et al., 2008). Each transporter consists of two cytoplasmic nucleotide-binding domains (**NBDs**) and two transmembrane domains (**TMDs**). The NBDs are highly conserved across the ABC families and contain motifs typical of ATP-binding sites, whereas the TMD structures vary, probably because they are adapted to a wide variety of substrates. In eukaryotes the C-terminal of each NBD is linked to a TMD. In some cases the functional unit is $(NBD\text{-}TMD)_2$ and, in others, the first TMD is covalently linked to the second NBD.

The Three-dimensional structures of several ABC transporters from prokaryotes have been determined

MsbA is a homodimeric ATP-binding cassette (ABC) exporter that is essential for most gram-negative bacteria to transport the lipid A hydrophobic moiety of the cytoplasmic membrane to the outer membrane. The lipid otherwise

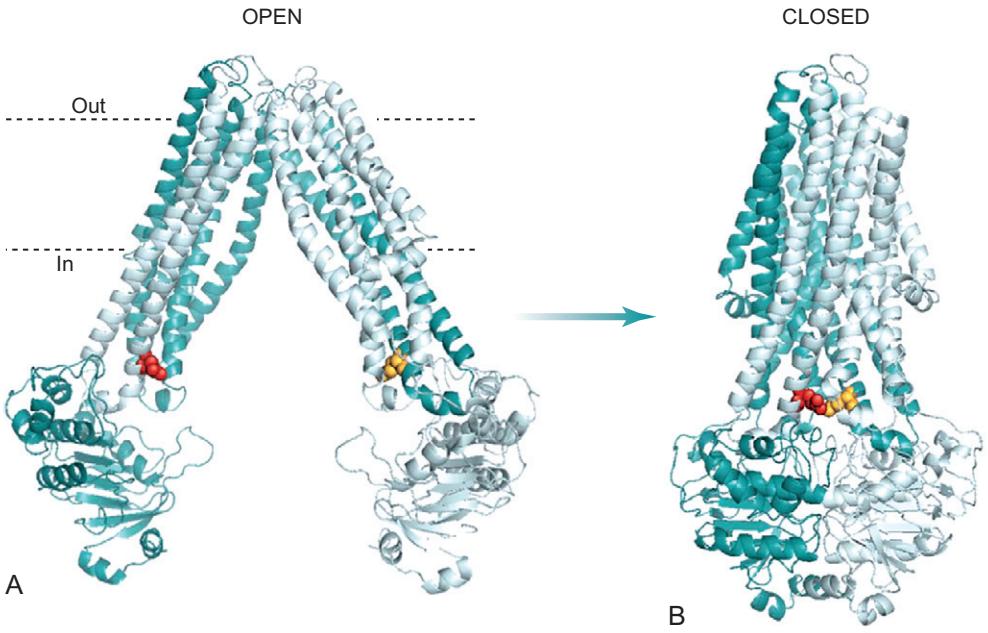

FIGURE 3-8 **Structure of homodimeric MsbA. A:** The "open-apo" crystal structure of the *E. coli* MsbA dimer in a nucleotide-free inward-facing conformation (PDB, 3B5W, full model) reveals residues E208C and E208C′ (marked in red and orange) predicted to be separated by more than 55Å (too wide for disulfide cross-linking). *In* and *Out* refer to the inside and outside of the plasma membrane, respectively. **B:** In the closed outward-facing AMP–PNP-bound crystal structure of *S. typhimurium* MsbA (PDB 3B60) in which the NBDs dimerize, this distance is only about 6Å (permitting thapsigargin disulfide cross-linking between E208C–E208C′). (With permission, from Fig. 1 of reference (Doshi, et al., 2010).)

would accumulate as an endotoxin. MsbA is also responsible for exporting many of the substrates of the human multidrug-resistance P-glycoprotein ABCB1. MsbA is a prototype ABC exporter for studies of the conformational movements that constitute the catalytic cycle of these ABC exporters. Figure 3-8 compares NBD structures from nearly identical MsbA transporters from *E. coli* and *S. typhimurium* that crystallized with their TMDs in open (Fig. 3-8A) and closed (Fig. 3-8B) conformations, respectively. Hypothetically, substrate would be acquired and transported as a result of the conformational cycle energized by its coupling to ATP hydrolysis (Doshi et al., 2010).

In the closed configuration (Fig. 3-8B), demonstrated by disulfide bridging between two CYS residues substituted for two GLU208 in the cytoplasmic extensions of transmembrane helices 4/4′, the two NBD domains bind to each other as a homodimer. As shown in Figure 3-9, nucleotide binds in the pocket formed between these two domains (Jones & George, 2004). In the open configuration (Fig. 3-8A), presumably coupled to ATP hydrolysis, the NBDs separate (Doshi et al., 2010; Jones & George, 2004).

ABCA1 translocates cholesterol and phospholipids outward across the plasma membrane

They are delivered first to the inner plasmalemma leaflet via vesicular pathways (Vance & Hayashi, 2010). This process occurs in astrocytes and developing neurons. Astrocytes also

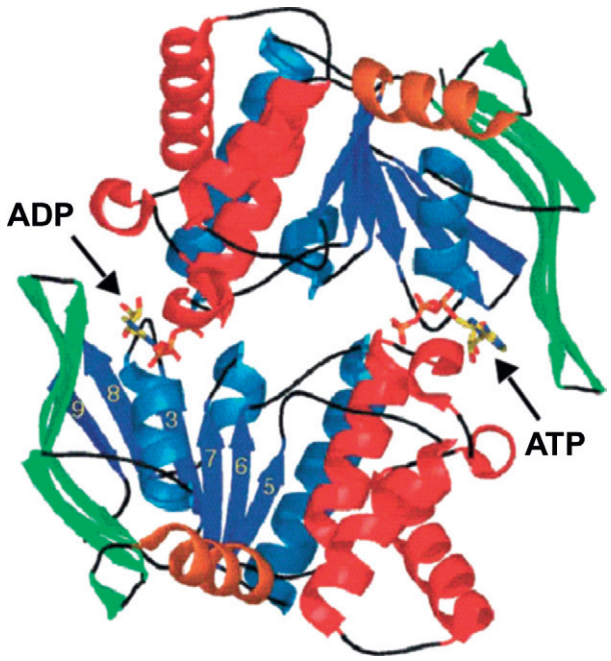

FIGURE 3-9 **Ribbon diagram of an NBD dimer (PDB 1f30).** β strands are depicted as arrows and α helices as coiled ribbons. The two nucleotides, shown as stick models, bind to form part of the interface that stabilizes the dimeric interaction. (With permission, from Fig. 5 of reference (Jones & George, 2004).)

secrete apoE. Extracellular apoE binds and interacts with ABCA1 to promote cholesterol and phospholipid efflux from cultured astrocytes through a mechanism that results in apoE-stabilized HDL-like particles (see Fig. 2-7). In the absence of apolipoprotein, ABCA1 is rapidly proteolyzed by calpain with a $T_{1/2}$ of only about 20 minutes in some cells. However, apolipoproteins protect ABC1A from calpain and increase its level of expression. Thus the interaction of apolipoprotein with ABCA1 is part of the regulatory process of cholesterol efflux. A closely related transporter, ABCA2, is in oligodendrocytes and is markedly upregulated during myelinization (Vance & Hayashi, 2010).

The multidrug-resistance proteins (MDR) can 'flip' amphipathic molecules

These molecules, including membrane phospholipids and sphingolipids, are flipped from the inner to the outer leaflet of plasma membranes and are instrumental in blood–brain barrier function (Ueno, 2009). One member, MDR3, can selectively transport phosphatidylcholine. MDR1 or 'P-glycoprotein' is the classic MDR, consisting of two cassettes within a single peptide chain. By pumping the drugs out of the cells, its elevated expression during chemotherapy can decrease the chemosensitivity of cancer cells. P-glycoprotein is expressed at high concentrations in the luminal membranes of brain capillaries and probably accounts for many drug-exclusion functions of the blood–brain barrier (Ueno, 2009). Adrenoleukodystrophy and Zellweger syndrome (Ch. 43) result from defects in two different genes that specify 'single-cassette' proteins targeted to peroxisomes.

SECONDARY ACTIVE TRANSPORT

Secondary transporters employ energy stored in ion gradients to transport other ions and molecules uphill. Many of these are symporters or antiporters linked to Na^+ or proton gradients (Fig. 3-1). However, the situation is often more complex, involving more than one ion gradient. A systematic nomenclature for secondary transporters is based on genomic analyses of the evolutionary relationships among those transporters that are members of a large 'solute carrier' family (SLC). Na-dependent glucose and neurotransmitter transport are discussed below. Na^+-dependent amino acid transport across cell membranes in general is subserved by several systems classified according to the structure of the amino acid. The reader is referred to a comprehensive review (Bröer, 2008).

Brain capillary endothelial cells and some neurons express a Na-dependent D-glucose symporter

SGLT1 (SLC5A1) was the first characterized of the large SLC5 family of Na^+-dependent symporters that transport various solutes and ions into cells (Vemula et al., 2009). In brain vascular endothelium, SGLT1 expression is limited to the luminal membranes of brain capillary endothelial cells. This suggests that SGLT1 and GLUT1 (glucose transporter 1) are both involved in glucose transport from blood into capillary endothelia, whereas glucose efflux from the endothelia into astrocytes and neurons depends primarily on GLUT1. The low affinity of GLUT1 for intracellular glucose (K_m ~25 mmol/l) may require SGLT1 on the luminal membrane to accumulate sufficiently high endothelial intracellular glucose to maintain an adequate rate of supply to the astrocytic endfeet.

Neurotransmitter sodium symporters (NSS) effect the recovery of neurotransmitters from synaptic clefts

The reuptake of neurotransmitters into the presynaptic cytosol and their storage in cytoplasmic vesicles is accomplished by the tandem actions of the sodium symporters in plasmalemma and the proton antiporters in the vesicle membranes. Sodium-dependent symporters mediate neurotransmitter reuptake from synaptic clefts into neurons and glia, utilizing energy from the Na^+ gradient across the plasmalemma. Proton-dependent antiporters concentrate neurotransmitters from neuronal cytoplasm into presynaptic vesicles, utilizing energy from the proton gradient across the vesicle membrane (Fig. 3-10C). The concentrating forces may be quite large, as in the case of glutamate illustrated in Figure 3-10C. The glutamate cytosol concentration is in the order of 1000-fold relative to the extracellular concentration and the intravesicular concentration is in the order of 20-fold relative to the cytosol. While the exact demand by neurotransmitter reuptake on cerebral energy metabolism is difficult to ascertain, the required subservient H^+- and Na,K-ATPase activities contribute substantially to the increased metabolic rate associated with neuronal activity (see Ch. 11).

There are two distinct subfamilies of neurotransmitter sodium symporters

The larger subfamily, SLC6, includes the Na^+,Cl^--dependent symporters for γ-amino butyric acid (GABAT), glycine (GLYT1,2), norepinephrine (NET), dopamine (DAT), and serotonin (SERT) (Torres et al., 2003). In this group, Cl^- is cotransported but, because in most cells it is passively distributed across the plasma membrane, chloride ion diffusion does not supply energy to the system. GABA reuptake is mediated by the secondary symporters, GABAT1–4 (SLC6A1–4), which are expressed in GABAergic neurons (see Ch. 18). Glycine symporters in the CNS are labeled GLYT1 and 2. GLYT2 is mainly expressed in glycinergic neurons and GLYT1 is found in glia and neurons. Of particular interest is the finding of GLYT1 on presynaptic glutamatergic nerve endings, where they interact with NMDA glutamatergic receptors. The smaller subfamily, SLC1, includes the Na^+-dependent glutamate symporters that are discussed later

The SLC6 subfamily of symporters for amino acid transmitters and biogenic amines is characterized by a number of shared structural features

The features shared across the subfamily are 12 predicted transmembrane helices with intracellular N- and C-terminals,

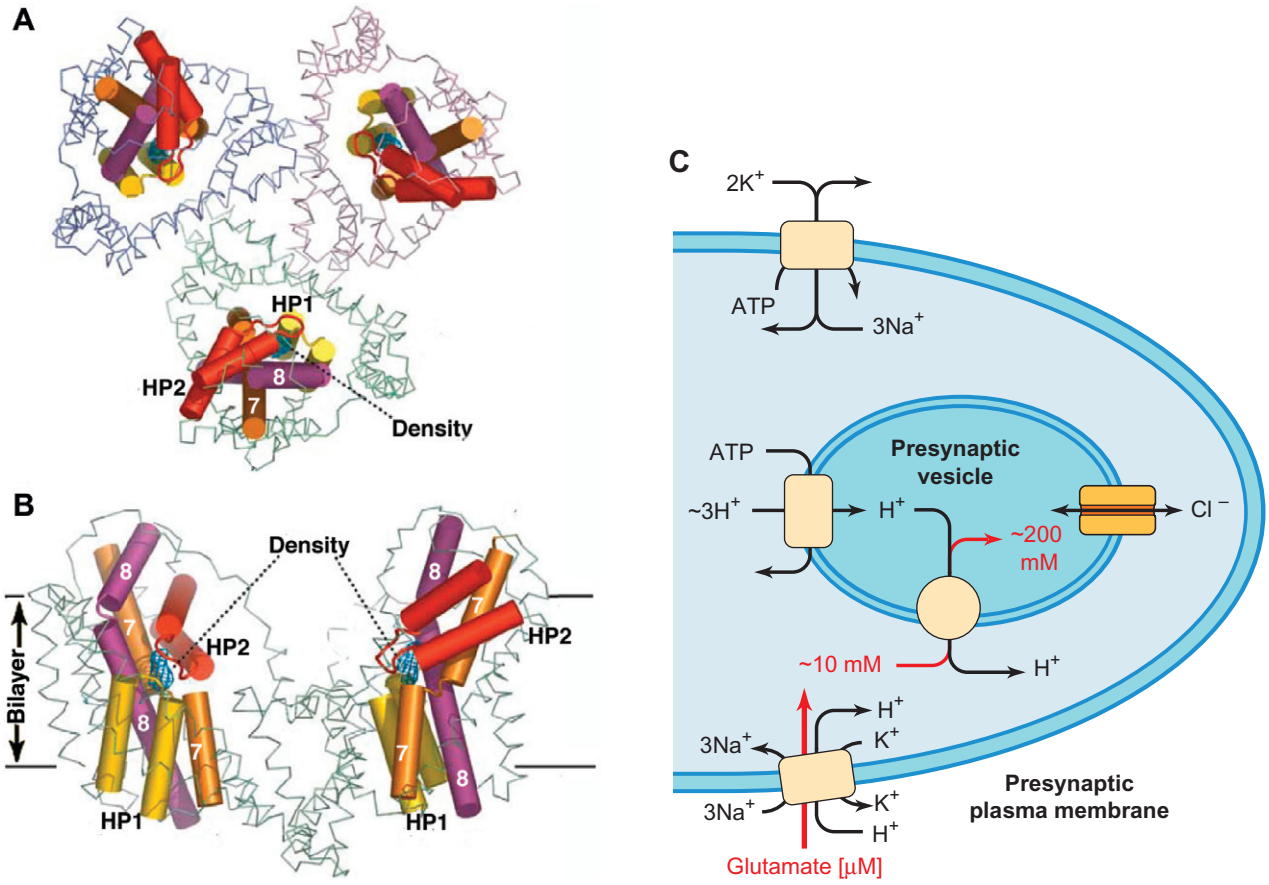

FIGURE 3-10 **Structure of a glutamate transporter.** This bacterial glutamate transporter provides the first high-resolution model of a glutamate transporter (Yernool, et al., 2004). The X-ray data indicate a trimeric structure. **(A)** A view of the trimer extracellularly and perpendicular to the bilayer. **(B)** A view from the bilayer plane. The trimer forms a deep bowl that allows extracellular solutes to access the extracellular glutamate-binding site located near the center of the bilayer plane. The helices represented by colored cylinders are those involved in the structure of the ionophoric sites, which involve transmembrane helices 7 and 8 and two re-entrant loops, HP1 (yellow) and HP2 (red), that enter from opposite sides of the bowl. The objects marked 'Density' appear to be glutamates bound at the ionophoric sites. The authors discuss this structure in terms of a model in which each bound glutamate is carried into the cytoplasm by obligatory coupling to the symport of 3 Na^+ and 1 H^+. (With permission from reference (Yernool, et al., 2004).) **(C)** Processes involved in neurotransmitter uptake and packaging at nerve endings. Concentration into the cytoplasm is achieved by means of Na^+ symporters with high affinity and specificity for the neurotransmitter in the plasmalemma. As indicated in red, μM extracellular glutamate is concentrated to 10 mM levels in cytoplasm and to about 200 mM in the vesicles. Synaptic vesicle membranes contain the V-ATPase proton pump (Fig. 3-7) and chloride channels, which together acidify the internal vesicle space and generate a proton gradient that drives the neurotransmitter-selective antiporters.

multiple N-glycosylation sites and several phosphorylation sites. Most of the current structural data relate to the biogenic amine transporters that are the major research targets for psychotherapeutic medicines and addictive substances such as cocaine, methylphenidate and amphetamine. The catecholamine and serotonin transporters are inhibited by a variety of drugs, both therapeutic and addictive. See Chs. 60 and 61 and (Eriksen et al., 2010; Wang & Lewis, 2010) for detailed reviews.

SLC1 proteins encompass glutamate symporters as well as some amino- and carboxylic-acid transporters expressed in bacteria

X-ray diffraction data have been obtained from crystals of one of these (Yernool et al., 2004) (Fig. 3-10). Analysis of multiple sequence alignments indicates that this molecule has a high degree of structural similarity to the glutamate transporters that are expressed in brain. Each subunit appears to contain an ionophoric site that can bind glutamate, probably in association with three Na^+ and one H^+. This site is located near the central plane of the bilayer and involves two of the eight transmembrane helices plus two 're-entrant loops', one from each side, which may act as 'access gates' that open sequentially to permit binding and dissociation within each transport cycle.

The glutamate symporters in brain are coded by five different but closely related genes, SLC1A1–4 and SLC1A6

There are several trivial names for each of the corresponding proteins. The symporters can all symport one Glu with three

Na^+ and one H^+, and antiport one K^+ within each cycle, but they differ in their cellular expression. The isoforms have different regulatory interactions and are expressed in different cell types. Being responsible for exquisite control of the access of the main excitatory transmitter in brain, glutamate, to specific synapses and for protection against excitotoxicity, coordination of the glutamate symporters with other synaptic structures and transporters is to be expected. Their density and distribution are regulated at transcriptional and post-translational levels. Astrocytes recover most of the synaptically released glutamate via the transporters GLT1/EAAT2 (aka SLC1A2) and GLAST/EAAT1 (aka SLC1A3). They convert much of this to glutamine, a reaction catalyzed by glutamine synthetase in the astrocytes, and the glutamine is recycled to neurons via the neuronal membrane Na^+-dependent amino acid transporter system (system A) (Blot et al., 2009). A mutant form of SLC1A2 has been associated with one form of amyotrophic lateral sclerosis (see Ch. 45). Knockout animals deficient in GLAST or GLT-1 have elevated extracellular glutamate and, in the case of mice, have lethal seizures (Jiang & Amara, 2010).

Failure of regulation of glutamate concentration in its synaptic, extracellular and cytosol compartments leads to critical pathology

While brain total glutamate concentration is about 10μmol/l, extracellular glutamate measured by in vivo microdialysis is normally only 3–4μmol/l. Glutamate within the synaptic cleft may be reduced by the glutamate transporter to 0.6μmol/l or less. Na,K-ATPase is linked to the glutamate transporters GLAST and GLT-1 representing an oligomolecular complex that functions as an integrated unit (Rose et al., 2009). Under depolarizing or anoxic conditions that deplete ATP, the membrane potential and the Na^+ and K^+ gradients can decrease to levels that cause the glutamate symporters to fail or to operate in reverse, producing 100- to 1,000-fold increases in extracellular glutamate, which is the condition known as *excitotoxicity* (see in Ch. 35).

Perisynaptic astrocytic transporters confine the extracellular free glutamate to receptors nearest to the release sites and prevent spill over onto adjacent synapses. The total concentration of glial transporters in some CNS synaptic regions is estimated to be sufficient to bind the glutamate content of three to five vesicles per synapse. This 'buffering', which occurs mostly in endfeet of astrocytes, is essential for reliable synaptic transmission of high-frequency signals.

Choline transporter: termination of the synaptic action of acetylcholine is unique among neurotransmitters

Acetylcholine action is terminated by hydrolysis rather than by transport (Ch. 13). Consequently, cholinergic neurons recover choline, rather than acetylcholine, via the high-affinity choline transporter CHT-1. A major fraction of these CHT-1 transporters reside in presynaptic vesicles and are only incorporated into the presynaptic membranes concurrently with the release of acetylcholine (Ferguson et al., 2003). This is a form of regulation somewhat analogous to that observed for the SLC6 family noted above. Structurally, CHT-1 is most similar to the Na^+-dependent glucose symporter and is classified within the SLC5 gene family.

Packaging neurotransmitters into presynaptic vesicles is mediated by proton-coupled antiporters

The V_0V_1-type primary transporters (H^+-ATPase) pump protons into the lumen of the vesicles. The protons then exchange for the cytosolic neurotransmitters via the vesicle antiporters.

Glutamate packaging antiporters are VGLUT-1, -2, and -3. VGLUT-1 and -2 are principally localized in glutamatergic neurons. They exhibit high specificity but low affinity (~1mmol/l) for cytoplasmic glutamate. They are saturated by the high concentration of cytoplasmic glutamate (~10mM). Some indication of the functional significance of different isoforms can be inferred from demonstrations that VGLUT2 is expressed in some dopaminergic neurons whereas VGLUT3 vesicles occur in some serotonergic and GABAergic interneurons. Glutamate released from such neurons may be acting on metabotropic presynaptic receptors as autoregulators. The ability of vesicles to concentrate glutamate is critical to life, as shown by the facts that VGLUT1 and VGLUT2 full knockout mice do not survive past weaning or birth (Wallén-Mackenzie et al., 2010).

The GABA-packaging antiporter VGAT (or VIAAT) was first identified by the characterization of a *Caenorhabditis elegans* mutant gene that produces a phenotype mimicking ablation of an identified GABA neuron. The mammalian ortholog of this gene functions in both GABAergic and glycinergic neurons. It is a proton antiporter and, like the VGLUTs, has relatively low affinity for its substrates, GABA and glycine.

The monoamine neurotransmitters (serotonin, norepinephrine, epinephrine and dopamine) are packaged into vesicles by the relatively nonspecific antiporters VMAT1 (SLC18A1) and VMAT2 (SLC18A2) (Eiden et al., 2004). Neuronal selectivity for a particular monoamine is determined by the particular biosynthetic enzymes expressed in the neuron. VMAT1 is predominantly distributed to the periphery for sequestration of epinephrine and norepinephrine and to melatoninergic cells of the pineal gland, where it concentrates serotonin. While VMAT2 is mainly in the CNS, where it concentrates dopamine, norepinephrine, epinephrine and histamine, it is also found in histaminergic cells of gastric epithelium and in beta cells of the pancreas. According to recent research with mammalian neurons and neuroendocrine cells, VMAT1 and VMAT2 uptake and storage of monoamines is subject to regulation by two Ca^{2+}-dependent activator proteins of secretion (*CAPS*1 and 2) linked to the heterotrimeric GTPase G_o (Brunk et al., 2009). Beside functioning in packaging of neurotransmitters, these vesicles may act as a protective mechanism to remove from the cytosol endogenous oxidizing substances such as dopamine and exogenous toxicants, such as $MPTP^+$ or methamphetamine, that produce oxidative stress (Guillot & Miller, 2009). Acetylcholine is packaged in synaptic vesicles of both central and peripheral neurons by the same antiporter, namely VAChT (SLC18A3) (Eiden et al., 2004).

GENERAL PHYSIOLOGY OF NEUROTRANSMITTER UPTAKE AND STORAGE

Presynaptic vesicles are estimated to each contain approximately 3,000–5,000 transmitter molecules. the internal volume of a vesicle is such that its internal transmitter concentration may be ~0.5mol/l. Vesicle contents may be released within less than 1ms and <0.02μm from the postsynaptic receptors. The volume within a synaptic cleft of ~1μm^3 will be ~1,000-fold greater than the vesicle volume, so that the instantaneous concentration is about 0.5mmol/l. Because only 3–4 free molecules within the volume of such a cleft are effectively ~1μmol/l, rapid inactivation of a postsynaptic receptor requires removal of essentially all of the transmitter. Glutamate transporters have affinity constants of more than 10μmol/l, and transport reaction cycles are slow relative to impulse rates. This problem appears to be solved by having an extremely high density of transporters in the astroglial processes immediately adjacent to CNS glutamatergic synapses. The densities of astroglial transporters in processes near glutamatergic synapses are estimated to be 5,000–10,000/μm^2 (Lehre & Danbolt, 1998); binding of glutamate to transporters is an important factor in the rapid (ms) removal of glutamate and thus the distribution of transporters can regulate the accessibility of glutamate to extrasynaptic receptors.

Cytoplasmic chloride ion must be transported outward to generate the Cl^- gradient that allows GABA- or glycine-gated channels to hyperpolarize neurons by admitting Cl^-. In mature neurons, this outward transport is mediated by a K,Cl symporter, KCC2. However, immature neurons, in contrast, accumulate chloride via an inward Na,Cl symporter, NKCC1. Thus, activation of $GABA_A$ channels in immature neurons is depolarizing (see Ch.18). The ratio of KCC2/NKCC1 expression increases with maturation in most neurons and can be regulated in mature neurons. For example, in suprachiasmatic neurons this ratio is controlled by clock genes and in other neurons by endocrine cycles.

In the 'normal' state of mature neurons, the K^+ diffusion potential is similar to the membrane resting potential and thus has little ability to energize KCC2. However, K^+ released during membrane depolarization may transiently activate KCC2 symport, lower cytoplasmic [Cl^-] and increase the inhibitory postsynaptic current through $GABA_A$ channels. Excitation elevates cytoplasmic [Ca^{2+}]. This elevation apparently upregulates KCC2 and is the basis of inhibitory 'coincidence detection,' which has been demonstrated in hippocampal neurons; this coincidence is that of excitatory impulses arriving simultaneously with $GABA_A$ channel activation at inhibitory synapses (Woodin, et al., 2003).

THE CATION ANTIPORTERS

Na,Ca exchangers are important for rapidly lowering high pulses of cytoplasmic Ca^{2+}

They can remove cytoplasmic Ca^{2+} up to 10 times faster than SERCA or PMCA pumps. The NCX exchange process is electrogenic, with three Na^+ exchanged for one Ca^{2+}. Three Na,Ca-antiporter isoforms are expressed in brain: NCX1 and NCX3 in neurons, NCX2 predominantly in glia.

Na,K-ATPase α subunits are coordinated with Na,Ca antiporters and Ca pumps

In tissue culture, astrocytes, hippocampal neurons and arterial myocytes all express α1 diffusely in their plasmalemma. In contrast, immunocytochemically stained α2 in astroglia and α3 in myocytes and neurons display reticular patterns that colocalize with the Na,Ca antiporter (NCX) patterns in each of the cell types (Fig. 3-11) (Juhaszova & Blaustein, 1997). These plasmalemma reticular patterns also coincide with the patterns of staining for junctional complexes in sarcoplasmic reticulum and on junctional complexes in endoplasmic reticulum of astroglia and neurons. These junctional complexes contain the smooth endoplasmic reticulum Ca pumps (SERCA).

Physical coupling among the Na,K-ATPase, NCX and sarcoplasmic reticulum calcium store was first demonstrated in smooth muscle cells. The N-termini of the α2 and α3 isoforms contain a common structural motif that allows these isoforms to be targeted to the plasmalemma microdomains overlying "junctional" sarco-endoplasmic reticulum (Juhaszova & Blaustein, 1997; Song et al., 2006).

The overall mechanism for regulation of cytosolic Ca^{2+} is complex

The overall mechanism depends on cell-specific interactions among the Na,K-ATPase, NCX, IP_3R, regulatory and structural proteins (see Figs. 3-5 and 3-6). In addition to calcium entry through NCX, recent research has demonstrated a direct interaction between the Na,K-ATPase α subunit and IP_3Rs in the ER (see Fig 3-5) (Chen et al., 2008; Liu et al., 2008). IP_3Rs are IP_3-gated Ca^{2+} channels that are linked by ankyrin-B to the plasmalemma Na,K-ATPase and NCX to form a functional calcium signaling domain in cardiac myocytes (Liu et al., 2008) (see also IP_3R in Chs. 23, 24).

In response to stimulation of G protein-coupled receptors or receptor-tyrosine kinases, either PLC-β or PLC-γ is recruited to the membrane and activated. The activated PLC in turn catalyzes the metabolism of PIP2, producing the second messenger IP_3 that binds to and opens IP_3Rs. A three-amino acid sequence (LEU-LYS-LYS) at the N-terminus of Na,K-ATPase α subunit is essential for its binding to IP_3R (Zhang et al., 2006).

Of many structural proteins, ankyrin is one of the most important for the formation of the calcium-signaling microdomains because it may use the Na,K-ATPase as an anchor to bridge the ER IP_3Rs to the plasma membrane receptors or channels/transporters. In addition to its interactions with NCX and IP_3R, the interactions of Na,K-ATPase with regulatory and structural proteins such as PLC and ankyrin play an important role in stabilizing the microdomain structure (Fig. 3-5) (Liu et al., 2008).

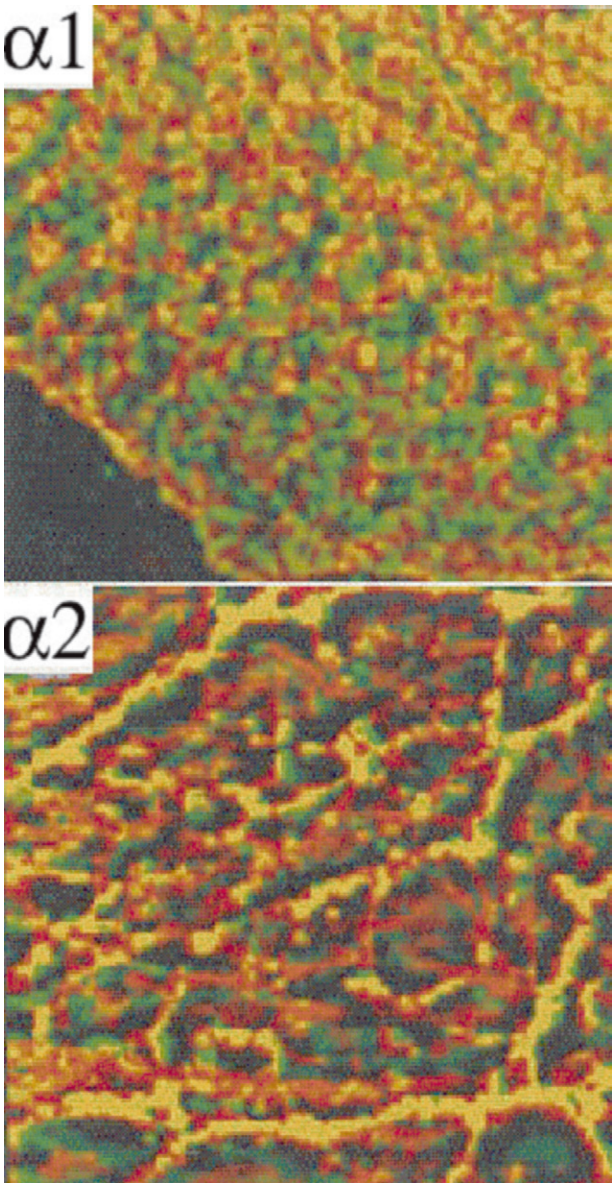

FIGURE 3-11 **Localization of Na^+ pump α1 and α2 isoforms in primary cultured astrocytes**. Cells were incubated with monoclonal antibodies specific for α1 or α2. The original fluorescent images were filtered by 'nearest neighbor' deblurring and the restored images compare portions of two astrocytes labeled with these antibodies. Both cells were later treated with a fluorescent dye, DiOC, to stain ER and mitochondria. When the respective antibody-labeled images (green) and DiOC-stained images (red) are superimposed, a yellow reticular pattern is observed where the ER and Na^+/K^+ pumps coincide. (Adapted with permission from Fig. 4 of Juhaszova & Blaustein (1997).)

THE ANION ANTIPORTERS

Anion antiporters comprising the SLC8 gene family all transport bicarbonate

CNS energy production derives almost entirely from glycolysis, resulting in a rate of metabolic CO_2 production nearly equal to the rate of oxygen consumption. In adult human brain, this is about 1.5 mmol/l per minute (Ch. 31). Because most neurons express only low concentrations of carbonic anhydrase, CO_2 diffuses out of neurons mostly unhydrated, but is converted to HCO_3^- before it enters the blood. The Cl,HCO_3 -anion antiporter (previously termed anion exchanger AE1(SLC4A1) and also known as band 3 protein) is a major protein of erythrocytes, where it mediates rapid uptake of HCO_3^- in exchange for Cl^- and functions in the reverse direction in lungs to exchange HCO_3^-. An isoform of this antiporter (AE3) is expressed in neurons, which suggests that significant anion exchange occurs across neuronal membranes (see in Alper, 2006 for references).

Intracellular pH in brain is regulated by Na,H antiporters, anion antiporters and Na,HCO_3 symporters

In principle the products of cerebral energy metabolism, primarily water and CO_2, may leave the brain and enter the circulation without disturbing the ionic balance. However processes such as incomplete oxidation, CO_2 hydration or Ca^{2+} influx can change local pH. Neuronal activity is accompanied by intracellular acidification and extracellular alkalinization. Cerebral metabolic rate decreases with decreasing pH so that, without adequate pH control, local metabolic deficits can be amplified and propagated (Siesjö et al., 1985). The pH response of astrocytes to membrane depolarization is opposite to the neuronal response: intracellular alkalinization and extracellular acidification occur. The neuronal acidification appears to involve antiporter exchange of HCO_3^- for Cl^-, whereas astrocytic alkalinization is probably mediated by a Na,HCO_3^- symporter. This is an oversimplification of CNS pH regulation in view of the fact that additional secondary transporters are involved in various aspects of local pH regulation.

Na,H antiporters (NHE) occur in synaptosomes, glia and neuroblastoma cells (Cai et al., 2008a). They are relatively inactive at neutral pH but with a decrease in intracellular pH they produce an efflux of protons at the expense of the Na^+ gradient. The NHE transport stoichiometry is 1:1. Activation by an internal pH decrement apparently results from protonation of a cytoplasmic site, which allosterically increases the affinity of the proton ionophoric site. In some cells, the NHE is under additional control by receptor mechanisms. Several growth factors and hormones produce transient cytoplasmic alkalinization, probably by mediating a protein kinase phosphorylation of the antiporter, which increases its affinity for cytoplasmic protons (Slepkov et al., 2007).

FACILITATED DIFFUSION: AQUAPORINS AND DIFFUSION OF WATER

Simple diffusion of polar water molecules through hydrophobic lipid bilayers is slow

In fact, the rate of diffusion would be much too slow to account for the observed rates of water flux across membranes in cells. Therefore, it was hypothesized that specific transporters or channels regulate rapid water movement across cell

membranes. Peter Agre and colleagues in 1992 isolated and identified the first member of a family of water transport molecules, now called aquaporin 1 (AQP1). Presently, this family is known to have 13 members found in mammals and about 150 in total found throughout all biological species. The aquaporin family consists of two subsets of channel proteins. One is the classical group, permeated only by water. The second group, aquaglyceroporins, is permeable to water or glycerol and to other small molecules such as urea (for references, see (Zeuthen, 2010)).

What do aquaporins do to lipid membranes? Cell membranes or synthetic lipid vesicles with normally low permeability to water will absorb water, swell and burst upon exposure to hypoosmotic solutions, if reconstituted with AQP1. The water permeability of membranes containing AQP1 can be about 100 times greater than that of membranes without aquaporins. The water permeability conferred by AQP1 (about 3 billion water molecules per subunit per second) is reversibly inhibited by Hg^{2+}, exhibits low activation energy and is not accompanied by ionic currents or translocation of any other solutes, ions or protons. Thus, the movement of water through aquaporins is an example of facilitated diffusion (Fig. 3-1), in this case driven by osmotic gradients.

Crystallographic and architectural data are available for AQP1 and AQP4

The 28 kDa monomeric protein of the prototypical AQP1 (Ueno, 2009) consists of six membrane-spanning α-helixes with both the carboxyl and amino terminals on the cytoplasmic surface and exists in membranes as a homotetramer. Each of the monomers, having roughly an hourglass configuration, contains a central water channel that can be accessed from either the extracellular or intracellular vestibules. A positively charged arginine residue near the pore obstructs protonated water (H_3O^+) and other cations, while two positively charged dipoles at the midpoint of the pore disrupt hydrogen bonds and prevent protons from passing through the pore (Zeuthen, 2010).

The aquaporins found in brain are AQP1, 4 and 9

AQP1 is in epithelial cells of choroid plexus (blood–ventricular CSF barrier); AQP4 is in basolateral surfaces of ependymal cells (ventricular CSF–brain barrier), astrocytic plasmalemma including perisynaptic lamellae (neuronal–astrocyte interface), subpial glial limitans (subarachnoid CSF–brain interface), and is most concentrated in astrocytic perivascular end-feet (blood–brain barrier); and AQP9, an aquaglycerol transporter, is in tanycytes and a small set of catecholaminergic neurons in the brainstem. Thusly, AQP4 provides the interface for water transport between brain and the systemic circulation at three locations, via perivascular endfeet, subpial glial limitans and ependyma.

AQP1 functions together with a number of transporters in choroid plexus to produce and regulate the formation and composition of CSF. In choroid plexus, AQP1 is localized to the apical surface of the epithelium where Na,K-ATPase, $Na^+/K^+/2Cl^-$ (NKCC) cosymporter and HCO_3^- channels are also situated. The basolateral membranes contain Cl,HCO_3 -and Na,H-antiporters. The Na,K-ATPase pumping Na^+ out of the epithelial apical surfaces into the ventricle provides the energy driving the osmotic gradient that extrudes water from epithelial cell apical surfaces through the AQP1 transporter.

In astrocytic perivascular endfeet membranes, AQP4 is anchored to the dystrophin complex of proteins

AQP4 is linked specifically to α-syntrophin (α-syn) that connects the cell cytoskeleton to the extracellular matrix (see Chs. 2 and 8). The anchoring proteins maintain the AQP4 in regular orthogonal arrays of particles (termed OAPs) that can be visualized by freeze-fracture electron microscopy as intramembrane particles in the endfeet. The AQP4 monomer exists in two forms: a full-length peptide and a truncated peptide starting with the 23rd amino acid (MET23) (Ho et al., 2009). The formation of OAPs requires α-syn and the shorter peptide that is the main constituent of the OAPs. The amount of OAPs is directly related to the ratio of short to long peptides (usually 3:1) and the OAPs exhibit greater water permeability than do the single monomers (Nicchia et al., 2010). Therefore, regulation of the ratio of short to long isoforms has the potential for regulating water transport and osmotic homeostasis through the BBB at the endfeet (Illarionova et al., 2010). The anchoring of AQP4 to α-syn is also critical to water permeability, since OAPs do not form in its absence and the astrocytic endfeet in mice that are α-syn null are defective in water transport (Amiry-Moghaddam et al., 2003).

AQP4 exists in astrocyte membranes and is coordinated with other proteins with which its function is integrated

In astrocytes from rat striatum and cerebellum, AQP4 interacts with the α1-catalytic subunit of Na,K-ATPase and with the mGluR5 receptor but not with astrocyte glutamate transporter GLT-1 (analogue of human EAAT2). The N-terminal segment 23–32 of AQP4 is critical for these interactions, while phosphorylation of the adjacent threonine 33 may be a regulatory factor. Thus, it is surmised that AQP4, Na,K-ATPase and mGluR5 function as an integrated oligomolecular complex in synaptic units (Illarionova et al., 2010).

AQP4 is found colocalized with inward rectifying potassium Kir4.1 and Kir5.1 channels in endfeet of brain astrocytes and retinal Mueller cells. In brain, homomeric Kir4.1 or heteromeric Kir4.1/5.1 are found at perisynaptic astrocytic processes, while only the heteromeric 4.1/5.1 channels are at the endfeet (Fig. 3-12) (Hibino et al., 2010). The Kir4.1 channels permit K^+ efflux or influx and are weakly inward rectifiers at the endfeet of astroglia or Mueller cells, thus facilitating the siphoning of K^+ away from perineuronal regions to the vitreous or capillary blood. The dense colocalization of AQP4 and Kir4.1 channels promotes the coordinated flux of water and K^+ from the periaxonal and perisynaptic regions through the astroglial

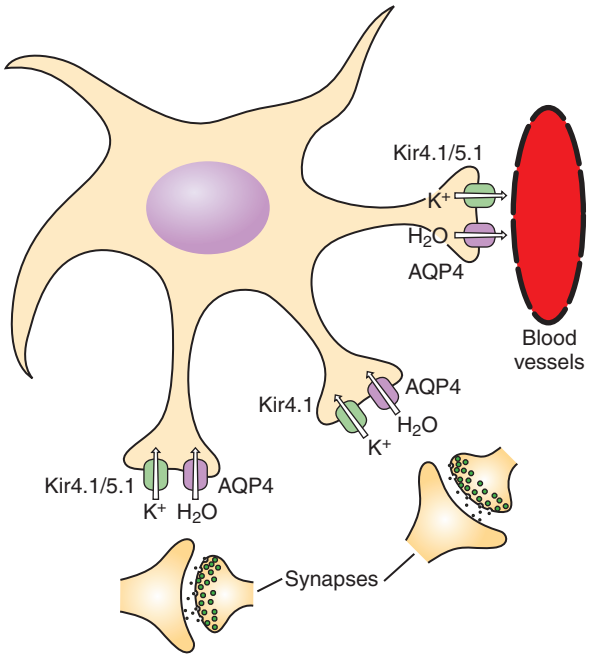

FIGURE 3-12 **Spatial buffering of K^+ by astrocytes**. Subsequent to its release from neuronal membranes during neural activity, the diffusion of K^+ toward the vascular bed is dependent on the coupled flow of water through the AQP4 channels and K^+ through the colocalized Kir4.1/5.1 channels in astrocytic, and finally through the perivascular astrocytic endfeet into the vascular bed in the CNS parenchyma and subpial glial limitans. Homomeric Kir4.1 and heteromeric 4.1/5.1 channels are located at perisynaptic astrocytic membranes, while only heteromeric 4.1/5.1 are found at the endfeet. (From Hibino, et al., 2010 with permission.)

syncytium and the endfeet into the systemic circulation (*spatial buffering*) (Masaki et al., 2010).

Rapid diffusion of K^+ and H_2O from Neuronal extracellular space by astroglia is critical to brain function

High extracellular K^+ depolarizes neurons. Neural activity discharges K^+, which, if not rapidly removed from the extracellular fluid, lowers the membrane potential of adjacent neurons. Normal neuronal activity can lead to elevations of 1–3 mmol/l in $[K^+]_e$ but, during epileptogenesis, concentrations can be three to four times higher (see Ch. 40). Usually, the upper limit of axonal impulse-conduction frequency is set by a conduction block that occurs when extracellular K^+ accumulates to a concentration of 25–30 mmol/l. In spreading depression, $[K^+]_e$ may reach 60 mM. Both neurons and astroglia are involved in K^+ uptake but their relative contributions are difficult to assess. It is clear that neurons accumulate K^+ almost exclusively by means of active transport, but the Na,K pump is slow relative to the channel-mediated K^+ release from neurons and to the rates of K^+ increase in the extracellular space. Moreover, the neuronal Na,K-pump is saturated at low $[K^+]_e$, making it an ineffective regulator for this purpose.

Glial processes invest nearly all extrasynaptic neuronal surfaces (see Ch. 1), and two of their principal functions are to regulate brain water and $[K^+]_e$. The extracellular space of the brain consists primarily of the 150–250 Å clefts separating glia and neurons. Astrocytes have higher K^+ permeability via Kir and NKCC channels and water permeability via AQP4 than most neurons, and astrocytes are extensively interconnected by gap junctions. K^+ efflux from a neuron can diffuse into the local glial cytoplasm, and compensatory K^+ efflux can occur from more distal reaches of the glial syncytium, where the $[K^+]_e$ is lower, as discussed in Figure 3-12. It should be noted that this 'spatial buffering' is a diffusion process that requires no energy input.

Short-term regulation of AQP4 may result from phosphorylation of either of two serine residues

Phosphorylation of Ser^{180}, stimulated by dopamine and catalyzed by PKC, results in *decreased* water permeability, while phosphorylation of Ser^{111}, activated by CaMKII, *increases* water permeability of AQP4. Ser^{111} is in a strategic position for control of channel gating. As mentioned above, agonists at mGluR1/5 receptors increase water permeability of astrocytes. These effects depend on NOS activation, suggesting that Ser^{111} can be phosphorylated by PKG via the pathway of mGluR-CaMKII-NO-cGMP-PKG signaling. Since mGluR5 activation increases the rate of hypoosmotic swelling in rat hippocampal slices, and since there is excessive release of glutamate in infarcted brain, this activation pathway may account for the experimental observations that ablation of AQP4 attenuates post-infarct brain edema (Gunnarson et al., 2008). By similar reasoning, one may expect dopamine agonists or PKC activators to reduce post-infarct cerebral edema (for references, see Yukutake & Yasui, 2010).

In neuromyelitis optica (NMO, or Devic's disease), the foci of edema and inflammation in optic nerve and CNS are associated with a specific IgG autoantibody produced against AQP4. The NMO-IgG reacts with a native extracellular epitope on the intact AQP4-anchoring protein complex in the astrocyte plasmalemma and initiates a complement-dependent inflammatory reaction while permeabilizing the membrane, as revealed in studies with cultured astrocytes (see references in Nicchia et al., 2010; Wingerchuk et al., 2007; and Box 39.).

FACILITATED DIFFUSION OF GLUCOSE AND MYOINOSITOL

Facilitated diffusion of glucose across the blood–brain barrier is catalyzed by GLUT-1, -2 and -3

These are products of the SLC2 gene superfamily. The SLC2 (solute carrier) superfamily consists of 12 glucose transporters (GLUT1–12) and one H^+-myoinositol cotransporter (HMIT or GLUT13). They all have 12 transmembrane segments with the N- and C-termini both on the cytoplasmic aspect and a specific N-linked oligosaccharide side chain on either the first or fourth extracellular loop.

The K_m of GLUT1 is 1–2 mmol/l for glucose influx and 20–30 mmol/l for efflux. A proposed transport mechanism evokes mutually exclusive glucose binding to sites internal and external to the plasmalemma; glucose binding to the extracellular side would induce a conformational change involving transport of the glucose. Possibly GLUT1 exists in membranes as a conformationally asymmetric dimer or tetramer (see (Simpson et al., 2008) for a comprehensive review).

GLUT1 is predominantly expressed in brain endothelial cells of the blood–brain barrier and on the astrocytic endfeet covering the abluminal surface of capillaries. GLUT1 is expressed also in Schwann cells and perineurium. GLUT1 is essential for glucose delivery from blood into brain through the astrocyte endfeet. Astrocytes metabolize glucose to lactate, which can be transferred into neurons via the monocarboxylate transporters MCT1 and MCT2 (see also Ch. 11). A hereditable mutation in the gene for GLUT1 results in a limited glucose supply to the brain and a syndrome of infantile epilepsy, acquired microcephaly and hypoglycorrhachia. A number of milder phenotypic variants have been described (Pascua, et al., 2004). It is noteworthy that, despite its vital dependence on an adequate glucose supply (Ch. 11), the brain relies for its supply of glucose on facilitators rather than active transport; therefore, supply is largely regulated by local blood flow (Ch. 11).

GLUT3 occurs in neurons, mainly in plasmalemma and to a lesser extent in nonsynaptic intracellular vesicles. GLUT2 and glucokinase are believed to be part of a glucose sensor system in certain neurons that modulate feeding behavior and regulate glucose uptake by peripheral tissues: their mRNAs are expressed in human ventromedial and arcuate nuclei of the hypothalamus (Simpson et al., 2008). GLUT8 is expressed in certain brain regions, including hippocampus, amygdala, olfactory cortex, hypothalamus, brainstem and posterior pituitary nerve endings. It is mainly observed in intracellular vesicles and may be translocated to the plasmalemma by as-yet-unknown regulatory stimuli. This endocytic recycling model of regulation may apply to others of the GLUT family. For example, GLUT4 in skeletal and cardiac muscle and adipose tissue occurs mostly in an intracellular vesicle compartment. Insulin activates its rapid translocation to the cell surface, resulting in increased cellular uptake of glucose. Exercise also regulates glucose uptake through trafficking of GLUT4 to the cell surface, but by an insulin-independent mechanism. Deficiencies in GLUT4 may underlie some variants of diabetes type 2 and insulin resistance as well as some skeletal or cardiac muscle disorders (Simpson et al., 2008).

HMIT is an H-coupled myoinositol symporter

High levels of its expression are observed in neurons and glia of hippocampus, hypothalamus, cerebellum and brainstem. Since myoinositol is a precursor for phosphatidyl inositol, which itself is a critical regulator of many neuronal processes (Ch. 23), HMIT regulation is possibly involved in various mood and behavior patterns that are affected by inositol metabolism and by pharmacologic agents that modify inositol metabolism (see Chs. 58, 60).

THERAPY OF BRAIN EDEMA: POTENTIAL PHARMACOLOGIC REGULATION OF AQUAPORIN 4

George J. Siegel

Inhibition or downregulation of AQP4 may aid in therapy of cytotoxic edema while activation or upregulation of AQP4 may aid in therapy of vasogenic brain edema (Saadoun & Papadopoulos 2010). In using mice null for AQP4, studies showed that AQP4 facilitates edema production manifested by astrocytic swelling under experimental conditions producing cerebral ischemia, hyponatremia, water intoxication or other hypoosmotic conditions, or meningitis. The AQP4 or α-syn null animals show less edema than do the respective wild types. In α-syn null animals, the deficit is in the number of OAPs, not in AQP4 monomers. The AQP4-null mice also exhibit prolonged seizures and delayed K^+ reuptake from the ECF during cortical spreading depression, which reveals the loss of K^+ flow through Kir4.1 channels and loss of water flow through AQP4 channels. These experimental paradigms show that AQP4 facilitates edema production manifested by astrocytic swelling under conditions that produce cerebral ischemia, hyponatremia, meningitis, water intoxication, or other hypoosmotic conditions. These are all examples of *cytotoxic edema.*

In contrast to the above-mentioned conditions, brain tumors or brain abscesses in animals null for AQP4 produced more edema than in the wild type, indicating that AQP4 facilitates removal of this type of edema. These are examples of *vasogenic edema*. Other examples of vasogenic edema in AQP4-null mice include intracerebral hemorrhage (Tang et al., 2010) and subarachnoid hemorrhage (Tait et al., 2010). This type of edema is produced independently of AQP4 (which has been ablated in the null animals) but requires AQP4 for its elimination through the three available AQP4 routes: perivascular endfeet around intraparenchymal vessels, the subpial glia limitans, and ependyma. In human edematous brain tumors, astrocytes adjacent to the tumor show increased expression of AQP4 throughout the astrocyte plasmalemma, not only at endfeet. This upregulation of AQP4 may be a protective mechanism.

Animals with overexpression of AQP4 in the endfeet develop more brain edema after water intoxication than do the wild type. Thus, AQP4 in the astrocytic endfeet is rate limiting for osmotic

THERAPY OF BRAIN EDEMA: POTENTIAL PHARMACOLOGIC REGULATION OF AQUAPORIN 4 (*cont'd*)

water movements across the blood–brain barrier. AQP4 is also important in spinal cord edema but fewer data are available (see references in Saadoun & Papadopoulos, 2010; Yukutake & Yasui, 2010 for further reading and methodology).

Recent reports along these lines are notable: arylsulfonamide AqB013 is an antagonist of AQP1 and AQP4 (Yool et al., 2010); phorbol myristate acetate, which is an activator of PKC, results in downregulation of AQP4 after cerebral ischemia in rats (Fazzina et al., 2010); in a study of water permeability of *Xenopus* oocytes expressing AQP4 and the vasopressin G protein-coupled receptor V1(a)R, the permeability was reduced in a vasopressin-dependent manner as a result of V1(a)R-dependent internalization of AQP4 (Moeller et al., 2009). It is notable that this interaction with vasopressin involves PKC activation and is reduced by mutation of Ser^{180} on AQP4 to alanine, as reported by Moeller and colleagues (Moeller et al., 2009), just as in studies with dopamine-dependent inhibition of mammalian astrocyte AQP4 described in this chapter. Edaravone, a free radical scavenger used in Japan for treatment of acute ischemic stroke, has been found to reduce the infarct area, improve neurologic deficit scores and markedly reduce AQP4 immunoreactivity and protein levels in the infarct area of rat brain after experimental cerebral ischemia (Kikuchi et al., 2009). The recently made discoveries of regulatory pathways for AQP4 that are sensitive to dopamine, glutamate and vasopressin provide strategies for continued investigation in the therapy of cytotoxic edema. Although the precise means of regulating water homeostasis in brain under all conditions are not yet fully understood, the available data point to certain processes meriting investigation regarding AQP4 function: channel gating, regulation by kinases, and OAP formation (Yukutake & Yasui, 2010).

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