Constitutive cycling: a general mechanism to regulate cell surface proteins

Stephen J. Royle[1,2]* and Ruth D. Murrell-Lagnado[2]

Summary

Cells can change their function by rapidly modulating the levels of certain proteins at the plasma membrane. This rapid modulation is achieved by using a specialised trafficking process called constitutive cycling. The constitutive cycling of a variety of transmembrane proteins such as receptors, channels and transporters has recently been directly demonstrated in a wide range of cell types. This regulation is thought to underlie important biological phenomena such as learning and memory, gastric acid secretion and water and blood glucose homeostasis. This review discusses the molecular mechanisms of constitutive cycling, its regulation by extracellular agents such as hormones and its misregulation in disease states. ***BioEssays*** **25:39–46, 2003.**

Introduction

The function of plasma membrane proteins can be up- or down-regulated by altering the number expressed at the cell surface. This type of regulation can be slow and involve either the synthesis of new protein or a change in the rate of degradation of existing protein. For some proteins, however, a rapid change in surface expression is achieved by having a pool of ready-synthesised molecules available in intracellular compartments and mechanisms for their rapid insertion into and retrieval from the plasma membrane. This pool resides in endosomal compartments and is formed by the constitutive internalisation of proteins from the surface. The proportion at the surface and in endosomal compartments will depend upon the relative rates of endocytosis and exocytosis (Fig. 1). Some proteins undergo rapid constitutive internalisation and subsequent slow recycling back to the surface and, therefore, under basal conditions, exist predominantly within intracellular compartments. A good example of such a protein is the glucose transporter GLUT4 that is expressed in muscle and fat cells. A rise in insulin levels causes a net translocation of this protein to the cell surface and it is via this mechanism that insulin promotes glucose uptake into muscle and fat cells. Other examples of proteins that constitutively cycle to and from the surface are the ionotropic receptors, AMPA, $GABA_A$ and P2X4. Within the central nervous system, changes in the rate of delivery and retrieval of AMPA receptors are important for the long-term potentiation (LTP) and depression (LTD) of synaptic transmission. Disruption of the normal cycling of proteins is known to underlie several disease states, for example the misregulation of epithelial sodium channels (ENaCs) in Liddle's syndrome. This review attempts to highlight the similarities in the trafficking of a number of transmembrane proteins by a variety of different cell types. The mechanisms involved in constitutive cycling (CC) and how it can be regulated will be discussed.

[1]Division of Neurobiology, MRC Laboratory of Molecular Biology, Cambridge.
[2]Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge.
Funding agency: Biotechnology and Biological Sciences Research Council and Wellcome Trust.
*Correspondence to: Stephen Royle, Division of Neurobiology, MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, UK. E-mail: sjr51@mrc-lmb.cam.ac.uk
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Abbreviations: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; AP-2, adaptor protein complex-2; AQP2, aquaporin-2; CC, constitutive cycling; CFTR, cystic fibrosis (CF) transmembrane conductance regulator; CME, clathrin-mediated endocytosis; DAT, sodium-dependent dopamine (DA) transporter; EM, electron microscopy; ENaC, epithelial sodium channel; ERC, endosomal recycling compartment; GABA, γ-amino butyric acid; GLUT4, glucose transporter-4; GRC, growth factor regulated channel; GST, glutathione *S*-transferase; LTD, long-term depression; LTP, long-term potentiation; NDI, nephrogenic diabetes insipidus; NMDA, *N*-methyl-D-aspartate; NSF, *N*-ethyl-maleimide (NEM)-sensitive fusion protein; SNARE, soluble NSF-attachment protein (SNAP) receptors; TGN, *trans*-Golgi network; VR1, vanilloid receptor.

Transmembrane proteins regulated by constitutive cycling

A list of proteins that undergo CC is shown in Table 1. In this review, we discuss the trafficking of ionotropic receptors (AMPA, $GABA_A$ and P2X4 receptors), active transporters (GLUT4 and H^+–K^+-ATPase), the cystic fibrosis transmembrane conductance regulator (CFTR), the water channel (AQP2) and the ENaC. Changes in the cell surface expression of these proteins are important for synaptic

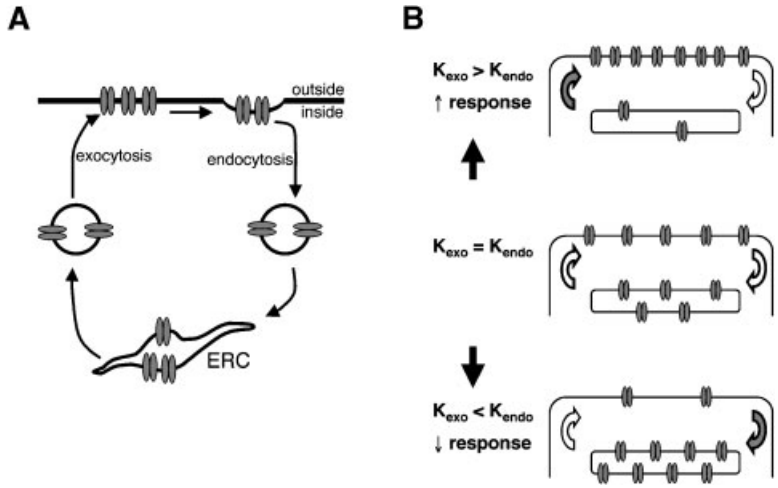

Figure 1. Schematic diagram of constitutive cycling and the bi-directional control of surface protein levels. **A:** Constitutive cycling represents the continual endocytosis of protein from the cell surface and its subsequent reinsertion into the plasma membrane. This scheme of CC is termed the simple model and extensions to this model are presented later. **B:** CC allows for the bi-directional control of the amount of protein at the cell surface. At steady state the relative rates of endocytosis and exocytosis are matched (middle part), the amount at the surface can be increased if the relative rate of exocytosis is increased with respect to endocytosis (upper part). The converse is also true; an increase in the relative rate of endocytosis with respect to exocytosis causes a decrease in the surface population (lower part). An increase in surface level relates directly to an increased response to a stimulus in the case of ionotropic receptors, or increased permeability and transport in the case of channels and transporters.

plasticity, water and glucose homeostasis and gastric acid secretion.

Forte and colleagues first proposed the "membrane recycling hypothesis" in the late 1970s to explain the control of gastric acid secretion.[1] Electron microscopy (EM) studies suggested that regulated exocytosis of proton pumps that were stored in tubulovesicles in parietal cells caused an increase in acid secretion.[2] In 1981, Wade and co-workers proposed a similar model to account for the regulation of water permeability by the kidney. Here, it was suggested that water channels stored in vesicles could be exocytosed in response to vasopressin.[3] Evidence to support this hypothesis accumulated[4] and later studies directly showed regulated trafficking of water channels in native cells and CC of AQP2 expressed in

Table 1. Transmembrane proteins that use constitutive cycling to regulate activity

Protein*	Type	Cell	Regulator**
AMPA receptor[64]	Glutamate-gated ion channel	Neurones	Neuronal activity, Insulin(↓)
$GABA_A$ receptor[66]	GABA-gated ion channel	Neurones	Insulin
P2X4 receptor[41]	ATP-gated ion channel	Neurones	ATP(↓)
AQP2[69]	Water channel	Collecting duct cells of kidney	Vasopressin
GLUT4[62]	Glucose transporter	Muscle and fat cells	Insulin
H^+–K^+-ATPase[2]	Proton pump	Gastric parietal cells	Secretagogue
CFTR[74]	Chloride channel/transporter	Epithelial cells	cAMP
ENaC[25]	Sodium channel	Epithelial cells	cAMP
H^+-ATPase[75]	Proton pump	Luminal cells of urinary bladder	CO_2
DAT[76]	Dopamine transporter	Neurones	PKC(↓)
GAT1[77]	GABA transporter	Neurones	PKC
KOR1[78]	Opioid receptor	Neurones	Dehydration
GRC[79]	Ion channel	Min-6 cells	IGF-1

*The transmembrane protein is shown together with the cell types in which cycling has been demonstrated. There are entries for ionotropic receptors, channels and transporters. These proteins have integral structures for activity (e.g. an aqueous pore for passage of ions) and thus their surface number is expected to be directly proportional to the response observed.
**In most cases, regulatory agents cause an increase in surface number and activity. Only where indicated (↓), does the agent cause a decrease.

cultured cells.[5,6] Similarly, in 1980, glucose transporters were first reported to be redistributed from intracellular compartments to the plasma membrane following insulin stimulation.[7,8] The GLUT4 member of the glucose transporter family was subsequently shown to account for the majority of glucose uptake in muscle and fat cells and it is now known that a number of divergent stimuli can change the levels of GLUT4 at the plasma membrane.[9] In the mid 1980s, Lynch and Baudry proposed that a similar mechanism could account for the changes in synaptic responses mediated by AMPA receptors seen during LTP and LTD.[10] Re-examination of this hypothesis was necessary following the discovery of so-called "silent synapses". A silent synapse is defined as an excitatory synapse that has an *N*-methyl-D-aspartate (NMDA) receptor response, but not an AMPA receptor response. The appearance of an AMPA receptor response at such synapses during LTP, with no change in the NMDA receptor response was evidence for a postsynaptic recruitment of AMPA receptors.[11] Several lines of evidence subsequently showed that AMPA receptors constitutively cycle into and out of the postsynaptic membrane and that the net insertion and internalisation of AMPA receptors can contribute to LTP and LTD, respectively.[11–13]

Mechanisms of endocytosis

Transmembrane proteins that undergo CC appear to be endocytosed by the clathrin-dependent pathway. The molecular mechanisms of clathrin-mediated endocytosis (CME) have been well studied,[14] and a diagram of how CME proceeds is shown in Fig. 2A. Transmembrane proteins that are to be internalised contain specific peptide sequences that are recognised by adaptor protein complexes that serve as a link to the clathrin coat. The main adaptor protein complex involved in recognising proteins at the cell surface is the AP-2 complex. AP-2 is a heterotetrameric assembly comprising large α and β2 subunits, a μ2 (medium chain) subunit and a σ2 (small chain) subunit (Fig. 2B). The complex has a core region made up from the μ2 and σ2 subunits together with the N-terminal regions of the large α and β2 subunits (Fig. 2C).[15] There are two "ear" regions comprising the C-terminal segments of the large subunits and these are linked to the core by hinge domains. The β2 subunit interacts with clathrin to induce its assembly at the plasma membrane.[16] The μ2 subunit is involved in recognising tyrosine-based sorting motifs within transmembrane proteins.[17,18] These motifs are generally of the form YXXϕ (single amino acid code where ϕ is an amino acid with a bulky, hydrophobic side chain and X is any amino acid). Other endocytotic signals, such as the NPXY or dileucine-based signals, have been shown to bind to sites other than μ2, namely the terminal domain of clathrin or the β2 subunits of AP-2, respectively.[19]

The intracellular domains of AMPA and $GABA_A$ receptors when expressed as fusion proteins with glutathione-*S*-transferase (GST) interact with AP-2.[20,21] A motif necessary for this interaction, however, has not yet been described for either receptor. The ionotropic P2X4 receptor interacts with AP-2 via its C terminus, and internalisation of this receptor is mediated by a non-canonical tyrosine-based sorting motif of the form YXXGL.[22] In the case of the water channel AQP2, no motif has yet been demonstrated for endocytosis. By contrast, ENaC has a short sequence in its C terminus (PPXYXXL) that is necessary for endocytosis.[23,24] It has been proposed that this sequence contains two overlapping motifs (PPXY and YXXL) that may serve to interact with Nedd4 and AP-2, respectively.[25] CFTR interacts with AP-2 via a canonical tyrosine-based sorting motif (YDSI) although other endocytotic motifs may be present in the C terminus.[26–29] There is also evidence for a role for AP-2 in the trafficking of H^+–K^+-ATPase. The beta subunit of this enzyme has in its N terminus a highly conserved tyrosine-based motif that is similar to the motif in the C terminus of the transferrin receptor (FRXY *versus* YXRF).[30] Mutating this tyrosine residue to alanine caused the H^+–K^+-ATPase to be expressed at high levels at the apical plasma membrane and this resulted in the continual secretion of HCl.[31] Several studies have looked for sequences involved in the retention of GLUT4 in endosomes and have uncovered an SLL motif that is necessary for internalisation.[32–35] Together these examples illustrate that there is not just one motif involved in the constitutive endocytosis of transmembrane proteins that undergo CC.

Following the recruitment of proteins to clathrin-coated pits via interactions with AP-2, the coated pit is "pinched off" by the action of dynamin (Fig. 2A). Dynamin is a large (100 kDa) GTP-hydrolysing protein (GTPase) whose function is essential for many vesicular trafficking events including CME.[36] A lysine-to-alanine mutation (K44A) in dynamin inhibits its GTPase activity and blocks endocytosis.[37,38] This dominant-negative mutant has been widely used to demonstrate that endocytosis of a protein is by a dynamin-dependent mechanism. Co-expression of dominant-negative dynamin with AMPA receptors,[39,40] P2X4 receptors,[41] ENaC,[24] and AQP2[42] has been shown to inhibit their internalisation. Several studies have also demonstrated the involvement of dynamin in GLUT4 endocytosis. Inhibition of dynamin function either by using dominant-negative dynamin or by microinjection of a peptide that interferes with dynamin function, resulted in a reduction in GLUT4 endocytosis and accumulation at the cell surface.[43–46]

Mechanisms of insertion

When compared with endocytosis, the molecular mechanisms underlying the insertion of transmembrane proteins undergoing CC have been less well studied. The available evidence suggests that, similar to other membrane fusion events, it involves SNARE (soluble NSF attachment receptor) proteins on both the vesicle and target membrane.[47,48] These

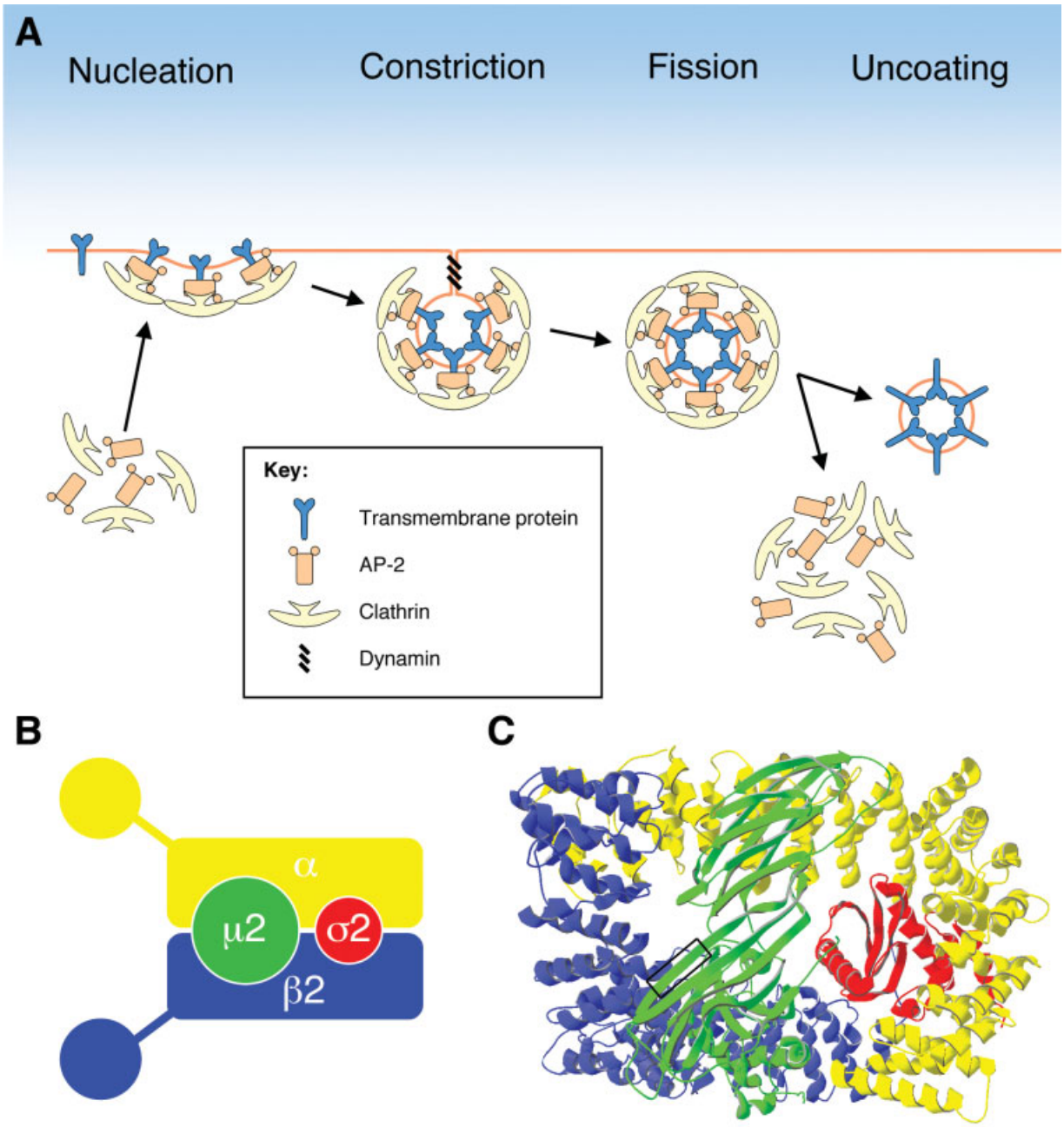

Figure 2. Clathrin-mediated endocytosis and the AP-2 complex. **A:** Schematic diagram to illustrate the internalisation of transmembrane proteins by the clathrin-mediated pathway. CME proceeds by four distinct stages: coated pit nucleation (comprising AP-2 recruitment, clathrin assembly, dynamin recruitment and invagination); coated pit constriction; coated vesicle scission; and coated vesicle uncoating (comprising clathrin release and AP-2 release). **B:** Schematic representation of the AP-2 complex. The α, β2, μ2 and σ2 subunits are shown in yellow, blue, green and red, respectively. **C:** Ribbon diagram to show the structure of the AP-2 core. The core region comprises the AP-2 complex without the "ear" domains[15] and each individual subunit is coloured as in B. The atomic co-ordinates for this structure can be found in the Protein Data Bank (PDB ID code 1GW5). The region of the μ2 subunit that binds YXXϕ signals[17] is highlighted by a black box.

proteins, respectively termed v- and t-SNAREs, interact to cause specific fusion of the vesicle at the correct area of target membrane.[47] The v-SNARE, synaptobrevin 2 (also known as VAMP-2) has been shown to be present on vesicles containing AQP2 channels, and the t-SNARE, syntaxin 4 is present on the apical plasma membrane of collecting duct cells.[49,50] Similarly, multiple studies have shown that insulin-stimulated insertion of GLUT4 into the plasma membrane requires synaptobrevin 2, syntaxin 4, SNAP-23 and other accessory proteins.[51] In addition, synaptobrevin 2 and syntaxin 3 have been identified in membranes rich in H^+–K^+-ATPase isolated from parietal cells.[52] A SNARE-mediated process also appears to be responsible for exocytotic insertion of AMPA receptors seen during LTP.[53] The *N*-ethyl-maleimide (NEM)-sensitive fusion protein (NSF), a key player in membrane fusion(47) has also been identified as an AMPA receptor binding partner.[54–56] Blocking this interaction causes a decrease in the number of AMPA receptors at the synapse.[54,56–59] The NSF-GluR2 interaction therefore either mediates AMPA receptor insertion or inhibits AMPA receptor endocytosis, and recent evidence supports the latter.[60]

In addition to SNARE proteins, a role for Rab proteins has been identified in the insertion of proteins undergoing CC. The Rab proteins represent a large family of GTPases that are involved in membrane fusion and exocytosis.[47] Rab11 and Rab25 were both identified on H^+–K^+-ATPase-enriched tubulovesicles from parietal cells.[2] Moreover, expression of dominant-negative mutant of Rab11a (N124I) in cultured parietal cells interfered with recruitment of H^+–K^+-ATPase to the plasma membrane.[61] In addition, a role for Rab4 in GLUT4 insertion has been demonstrated.[62] In conclusion, many of the molecular players in vesicle membrane fusion appear to be involved in the insertion of proteins undergoing CC.

Energetic considerations for constitutive cycling

CC is an energy-expensive process because both CME and exocytosis require hydrolysis of GTP and ATP, and there is also no energy gain. A biological system that occurs in a cycle and only expends energy is referred to as a 'futile cycle'. Certain enzymatic pathways undergo futile cycling and it has been proposed that the cyclical conversion of chemicals by two enzymatic reactions increases the sensitivity of the system because a small change in the rate of either reaction causes a large change in net flux.[63] Thus the energetic cost is the necessary expense for running an extremely sensitive system. CC operates in a similar way: the engine is kept ticking over and this allows for the throttle to go up or down at a moment's notice. This operation avoids the lengthy process of protein synthesis and allows large changes in cell signalling to occur on a rapid time-scale.[12,64]

Regulators of surface number

Several agents, including peptide hormones, can cause a change in the number of proteins at the cell surface (see Table 1). In the simple CC model, a regulator can cause an increase in protein at the cell surface by decreasing endocytosis and/or increasing exocytosis (Fig. 1). The increase in surface expression of GLUT4 by insulin has been shown to be due to a 2- to 3-fold decrease in endocytosis and a 10- to 20-fold increase in exocytosis.[62,65] There is evidence that the endpoint of the signalling pathways activated by insulin can result in inhibition of dynamin function, release of GLUT4-containing vesicles from a retention organelle and facilitation of the exocytotic machinery.[9,51,62] Insulin also causes an increase in the insertion of $GABA_A$ receptors,[66] suggesting a common mechanism between the regulation of this receptor in neurones and GLUT4 regulation in muscle and fat cells. Application of insulin, however, stimulates the removal of AMPA receptors from central synapses,[40] suggesting that its actions are specific to the control of individual proteins rather than a general effect on endocytotic mechanisms. Regulation of the surface expression of a transmembrane protein by a hormone such as insulin ensures that changes occur in a large number of cells simultaneously and also in cells at different locations in the body, such as muscle and fat cells. Another example is vasopressin, which causes a decrease in water excretion by the animal by promoting the surface expression of AQP2 in the collecting ducts of several thousand nephrons in the kidney.

Extensions to the constitutive cycling model

The simple model of CC is depicted in Fig. 1 and a variation of this model, which includes a retention apparatus in parallel to the ERC is shown in Fig. 3. This apparatus can sequester protein-laden vesicles away from the constitutive exocytotic pathway. Agents that allow the vesicles to be released back into the insertion pathway cause an increase in surface expression. For GLUT4, the retention apparatus has been proposed to be composed of the *trans*-Golgi network (TGN).[67] It remains to be determined how reciprocal traffic between recycling endosomes and the TGN occurs and how this pathway can be regulated. The subunit-specific trafficking of AMPA receptors is a good example of the use of both simple and retention CC pathways, within a receptor family. GluR2/3 receptors continually cycle between the plasma membrane and the ERC while GluR1/2 receptors exist in a large intracellular pool that is available for activity-dependent insertion.[64,68] Thus the concurrent use of simple CC for GluR2/3 receptors and retention CC for GluR1/2 receptors may underlie the differential regulation of AMPA receptor subtypes.

Diseases associated with constitutive cycling

The examples presented here show that CC of certain transmembrane proteins is required to maintain homeostatic

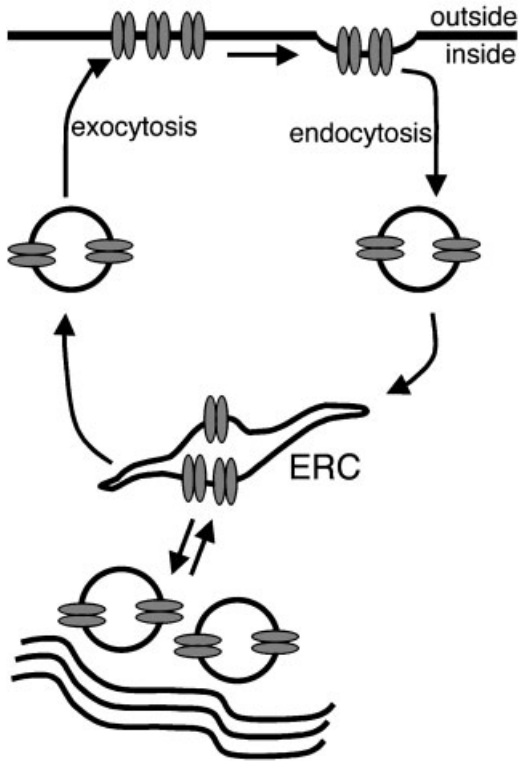

Figure 3. Extension to the simple constitutive cycling model. An extension to the simple CC model (depicted in Fig. 1) is shown here. The 'retention model' differs from the simple model in that protein-laden vesicles are retained in an organelle that is separate from the recycling apparatus. Vesicles can be freed to enter the recycling process in response to the stimulus.

biological systems. It follows therefore that interference of the CC of these proteins would upset homeostasis and lead to pathophysiological states. To illustrate this point, four examples are given here. First, the abnormal processing of water is the cause of a variety of pathophysiological conditions. Some of these conditions are due to altered abundance or targeting of AQP2.[69] For example, an autosomal dominant form of nephrogenic diabetes insipidus (NDI) is the result of mutations in the AQP2 gene that do not allow the proper trafficking of the channel to the cell surface.[70] Similarly, forms of NDI have been shown to be due to interference of vasopressin signalling in the kidney that prevent an increase in surface AQP2 levels in response to vasopressin.[69] Second, in Liddle's syndrome, which is a hereditary condition typified by high blood pressure, mutations in the PY motifs of ENaC subunits result in decreased channel internalisation.[23–25,71,72] Third, the disease cystic fibrosis (CF) is caused by mutations in the gene encoding CFTR, some of which alter the trafficking of the channel or prevent its exit from the endoplasmic reticulum.[73] Fourth, transgenic mice expressing the beta subunit of H^+–K^+-ATPase with a mutation in a tyrosine-based endocytotic motif have a high density of proton pumps in the apical membrane of parietal cells of the stomach. As a result, the mice exhibit hypersecretion of gastric acid that leads to ulceration and a hypertrophic gastropathy that resembles Ménétrier's disease.[31]

Concluding remarks

In this review, we have highlighted some of the similarities in the specialised trafficking of a variety of transmembrane proteins whose cell surface number can be rapidly upregulated or downregulated. We are beginning to understand the roles of trafficking proteins, such as adaptor complexes and SNARE proteins in CC; however, it is not known why only certain proteins undergo CC whereas others require activation prior to endocytosis and some do not recycle back to the plasma membrane. Structural data indicates that proteins interact with the μ2 subunit of AP-2 when they are presented in a dimeric form.[17] The proteins that undergo CC described here are mostly multimeric and so they could be recognised in a manner similar to proteins that have dimerised upon activation. In addition, recent evidence suggests that the AP-2 complex is not competent to receive an endocytotic signal and must undergo a conformational change before the μ2 subunit can contact tyrosine-based motifs.[15] If this is the case then, in cells expressing proteins that undergo CC, a greater proportion of the cellular AP-2 is in the active conformation. Clearly, a better understanding of how CC occurs and how it can be regulated is needed if we are to intervene in trafficking processes for therapeutic gain.

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