Module 5

OFF Mechanisms

Synopsis

Signalling pathways are composed of the ON mechanisms that generate internal signals and the OFF mechanisms that remove these signals as cells recover from stimulation. Most attention will be focused on how second messengers and their downstream effectors are inactivated. The second messengers cyclic AMP and cyclic GMP are inactivated by phosphodiesterase (PDE). Inositol trisphosphate (InsP₃) metabolism is carried out by both inositol trisphosphatase and inositol phosphatases. Diacylglycerol (DAG) metabolism also occurs through two enzyme systems, DAG kinase and DAG lipase.

In the case of Ca²⁺ signalling, recovery is carried out by the Ca²⁺ pumps and exchangers that remove Ca²⁺ from the cytoplasm. The mitochondria also play an important role in Ca²⁺ homoeostasis. Many of these second messengers activate downstream effectors through protein phosphorylation, and these activation events are reversed by corresponding protein phosphatases.

Protein phosphatases

It has been estimated that the human genome encodes approximately 2000 protein kinases that phosphorylate an enormous number of intracellular proteins, many of which function in cell signalling. There is an equally impressive array of protein phosphatases that are responsible for removing these regulatory phospho groups. These protein phosphatases can be divided into two main groups: the protein tyrosine phosphatases (PTPs) and the protein serine/threonine phosphatases.

Protein tyrosine phosphatases (PTPs)

It has been estimated that tyrosine phosphorylation accounts for less than 0.1% of all the protein phosphorylation in cells. Nevertheless, this small amount of phosphorylation is critical because it is involved in some very important signalling systems, and particularly those concerned with regulating cell growth and development. The fact that the level of tyrosine phosphorylation increases 10–20-fold when cells are stimulated by growth factors or undergo oncogenic transformation highlights the importance of protein tyrosine phosphatases (PTPs) in signal transduction. Protein tyrosine phosphatase structure and function reveals that these enzymes belong to a large heterogeneous family that functions to dephosphorylate phosphotyrosine residues with a high degree of spatial and temporal precision. The PTP superfamily can be divided into classical protein tyrosine phosphatases and dual-specificity phosphatases (DSPs).

Protein tyrosine phosphatase structure and function

The protein tyrosine phosphatase (PTP) superfamily is a heterogeneous group of enzymes with widely divergent structures. They can be divided into the classical PTPs and the dual-specificity phosphatases (DSPs). The former can be divided further into the non-transmembrane PTPs and the receptor-type PTPs. What all the phosphatases have in common is a signature motif (H-C-X-X-G-X-X-R) located in the PTP domain that is responsible for its catalytic activity. The different structural elements [e.g. Src homology 2 (SH2), PDZ and immunoglobulin-like domains] that flank this PTP domain function to regulate enzyme activity and to position the enzyme in the right location near its specific substrates. These structural elements are described in more detail when the individual enzymes are described.

All PTPs utilize the same catalytic mechanism during which the phosphate on the substrate is first transferred to the cysteine residue in the signature motif before being hydrolysed by water to release the phosphate anion. During redox signalling, this cysteine is oxidized, resulting in a decrease in the activity of the PTPs. Since the latter are normally expressed in great excess over the corresponding kinases, an oxidation-induced inhibition of phosphatase activity would greatly enhance the flow of information down those signalling cascades that rely on tyrosine phosphorylation, such as the mitogen-activated protein kinase (MAPK) signalling pathway and the Ca²⁺ signalling pathway...
pathway. In the case of the latter, a positive-feedback mechanism operates between the ROS and Ca^{2+} signalling systems (Module 2: Figure ROS effects on Ca^{2+} signalling).

Classical protein tyrosine phosphatases

The classical protein tyrosine phosphatases are composed of two main groups, the non-transmembrane protein tyrosine phosphatases and the receptor-type protein tyrosine phosphatases (Module 5: Figure tyrosine phosphatase superfamily).

Non-transmembrane protein tyrosine phosphatases

The non-transmembrane protein tyrosine phosphatases (PTPs) are a heterogeneous family that share similar PTP domains, but have additional elements that determine both their location and their function within the cell. The following are some of the major members of the non-transmembrane PTPs:

- Protein tyrosine phosphatase 1B (PTP1B)
- T cell protein tyrosine phosphatase (TC-PTP)
- Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-1 (SHP-1)
- Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-2 (SHP-2)

Protein tyrosine phosphatase 1B (PTP1B)

Protein tyrosine phosphatase 1B (PTP1B) has a typical protein tyrosine phosphatase (PTP) domain at the N-terminus and a regulatory region at the C-terminus. The latter contains a hydrophobic region that targets the enzyme to the cytoplasmic surface of the endoplasmic reticulum (ER). Despite this localization to the ER, some of the main substrates of PTP1B are the tyrosine kinase receptors, e.g. the epidermal growth factor (EGF) receptor and insulin receptors, and the non-receptor tyrosine kinase c-Src. PTP1B also acts on components of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway, such as STAT5a and STAT5b.

PTP1B plays a role in stabilizing cadherin complexes by dephosphorylating the phosphotyrosine residues on β-catenin. In order to bind to cadherin, PTP1B must be phosphorylated on Tyr-152 by the non-receptor protein tyrosine kinases Fer (Module 6: Figure classical cadherin signalling).

T cell protein tyrosine phosphatase (TC-PTP)

T cell protein tyrosine phosphatase (TC-PTP) has a similar structure to PTP1B, but operates on a different set of substrates. It exists as two alternatively spliced forms that differ with regard to the structure of the C-terminus. TC-48 has a hydrophobic domain resembling that of PTP1B and is similarly located in the endoplasmic reticulum (ER). On the other hand, TC-45 lacks the hydrophobic residue but has a nuclear localization signal (NLS) that directs it into the nucleus. When cells are stimulated with epidermal growth factor (EGF), the TC-45 leaves the nucleus and interacts with the EGF receptor complex, where one of its targets appears to be Shc.
**Module 5: Protein tyrosine phosphatase catalysis**

The catalytic mechanism of protein tyrosine phosphatases.

The signature motif, located at the bottom of a deep catalytic cleft, contains three important residues (cysteine, aspartate and glycine) that are necessary for the catalytic process. A. The peptide containing the phosphotyrosine (pTyr) residue enters the cleft where the cysteine residue initiates a nucleophilic attack. The aspartate residue has a critical role in protonating the phenolate leaving group in the substrate. B. Once the phosphohistidine has been transferred to the cysteinyl group, the substrate leaves the enzyme, and the final step is to hydrolyse the phosphate. The co-ordination of a water molecule to the glycine residue favours the hydrolysis of the phosphoryl residue. The nucleophilicity of the water molecule is increased by the abstraction of a proton by the aspartate residue. Once the phosphate has been removed, the active site is ready to hydrolyse another phosphotyrosine residue. Reproduced from Pannifer, A.D.B., Flint, A.J., Tonks, N.K. and Barford, D. (1998) Visualisation of the cysteinyl-phosphate intermediate of a protein-tyrosine phosphatase by X-ray crystallography. J. Biol. Chem. 273:10454–10462, with permission from the American Society for Biochemistry and Molecular Biology; see Pannifer et al. 1998.

**Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-1 (SHP-1)**

As their name implies, the Src homology 2 (SH2) domain-containing protein tyrosine phosphatases (SHPs) have two N-terminal SH2 domains (Module 6: Figure modular protein domains). There are two SHPs (SHP-1 and SHP-2), which have similar structures (Module 5: Figure structure of the SHPs). These SHPs must not be confused with the SH2 domain-containing inositol phosphatases (SHIPs), which form a subgroup of the Type II inositol polyphosphate 5-phosphatases, even though these two types of phosphatases often end up exerting very similar effects on cells.

Even though SHP-1 and SHP-2 are highly related structurally, they have very different functions. The primary function of SHP-1 is to inhibit signalling pathways that use tyrosine phosphorylation to transmit information. Many of its actions are directed against signalling systems in haematopoietic cells. It attaches itself to the signalling complexes via its SH2 domains, thereby enabling the protein tyrosine phosphatase (PTP) domain to dephosphorylate the phosphotyrosine residues involved in the process of signal transduction. Alternatively, SHP-1 is drawn into these signalling complexes through an attachment to various inhibitory receptors, particularly those that act to inhibit antigen and integrin receptor signalling. For example, SHP-1 is associated with the FcεRII receptors that inhibit the FcεRI receptors in mast cells (Module 11: Figure mast cell inhibitory signalling).

SHP-1 participates in an important feedback loop that exists between the reactive oxygen species (ROS) and Ca\(^{2+}\) signalling pathways (Module 2: Figure ROS effects on Ca\(^{2+}\) signalling).

**Protein tyrosine phosphatase α (PTPα)**

Protein tyrosine phosphatase α (PTPα) functions in the activation of the non-receptor Src family, where it removes the inhibitory phosphotyrosine residue.

**Receptor-type protein tyrosine phosphatases**

Receptor-type protein tyrosine phosphatase (RPTPs) have a transmembrane domain that retains them within the plasma membrane. Even though these enzymes are described as receptor-type, the nature of the ligand is poorly defined. Many of them have features of cell adhesion molecules and may thus be activated by cell-surface molecules embedded in neighbouring cells. This seems to be the case for RPTPs, which form homophilic interactions as they bind to identical molecules on opposing cells. The following are some of the major members of the RPTPs:

- **CD45**
- **Protein tyrosine phosphatase α (PTPα)**
- **Leucocyte common antigen-related (LAR)**

**CD45**

CD45 is a typical transmembrane protein tyrosine phosphatase (PTP) (Module 5: Figure tyrosine phosphatase superfamily). It has a highly glycosylated extracellular domain, and the cytoplasmic region has two PTP domains, but the second is catalytically inactive. CD45 has a critical function in T cell signalling, where it contributes early in the signalling cascade by activating Lck, which is a T cell receptor transducer (Module 9: Figure TCR signalling). It acts by dephosphorylating the phosphate on Tyr-505, which opens up the molecular structure of Lck so that it can begin to phosphorylate ζ-associated protein of 70 kDa (ZAP-70).

**Protein tyrosine phosphatase α (PTPα)**

Protein tyrosine phosphatase α (PTPα) functions in the activation of the non-receptor Src family, where it removes the inhibitory phosphotyrosine residue.
Structural organization of the Src homology 2 (SH2)-domain-containing protein tyrosine phosphatases (SHP-1 and SHP-2).
The two mammalian Src homology 2 (SH2) domain-containing protein tyrosine phosphatases (SHPs) have very similar structures. The main features are the PTP domain and the two N-terminal SH2 domains. The C-terminal region has two tyrosine (Y) residues, which in the case of SHP-2 are separated by a prolyl-rich domain.

Leucocyte common antigen-related (LAR)
The leucocyte common antigen-related (LAR) protein tyrosine phosphatase (PTP) has a number of specific developmental functions, such as a role in the terminal differentiation of alveoli in the mammary gland, as well as in development within the forebrain and hippocampus.

Dual-specificity phosphatases (DSPs)
As their name implies, these dual-specificity phosphatases (DSPs) are unusual in that they can dephosphorylate both phosphotyrosine (pTyr) and phosphoserine/phosphothreonine (pSer/pThr) residues. The following are some of the major members of the dual-specificity phosphatase family:

- Mitogen-activated protein kinase (MAPK) phosphatases (MKPs)
- Cdc25

Mitogen-activated protein kinase (MAPK) phosphatases (MKPs)
The family of mitogen-activated protein kinase (MAPK) phosphatases (MKPs) contains ten members (Module 2: Table MAPK signalling toolkit) that have specific functions in reversing the phosphorylation events responsible for the mitogen-activated protein kinase (MAPK) signalling pathway. One of the last events of this signalling cascade is the phosphorylation of the MAPKs by the dual-specificity MAPK kinases, which add phosphates to both tyrosine and threonine residues. During the recovery phase, these phosphates are removed by the MAPK phosphatases (Module 5: Figure dual-specificity MKP).

Some of the MAPK phosphatases are expressed constitutively, whereas others are actively induced when cells are stimulated, thus setting up a negative-feedback loop. An example of such a negative-feedback loop is evident for the extracellular-signal-regulated kinase (ERK) signalling pathway (Module 2: Figure ERK signalling). Another characteristic of these phosphatases is that they are often highly specific for particular targets. A good example of this specificity is illustrated by MAPK phosphatase-3 (MKP-3), which acts specifically to dephosphorylate ERK2.

Certain neurons, such as the medium spiny neurons in the striatum, express a striatal-enriched protein tyrosine phosphatase (STEP), which plays a highly specific role in regulating the neuronal MAPK signalling pathway (Module 10: Figure medium spiny neuron signalling). In response to N-methyl-aspartate (NMDA) stimulation, the increase in Ca²⁺ acts on calcineurin (CaN) to dephosphorylate and activate STEP, which then limits the duration of phospho-ERK signalling. By contrast, elevations in Ca²⁺ induced by voltage-operated channels (VOCs) or the release of internal Ca²⁺ have no effect, indicating a tight association between NMDA receptors and STEP.

Cdc25
The human genome contains three CDC25 dual-specificity enzymes (Cdc25A, Cdc25B and Cdc25C) (Module 9: Table cell cycle toolkit). This enzyme was
Mode of action of dual-specificity mitogen-activated protein kinase (MAPK) phosphatase.

Extracellular-signal-regulated kinase 2 (ERK2), which is one of the main components of mitogen-activated protein kinase (MAPK) signalling, is phosphorylated on threonine (T) and tyrosine (Y) by MEK1/2, a dual-specificity MAPK kinase (Module 2: Figure ERK signalling). These phosphorylation events are reversed by MAPK phosphatase-3 (MKP-3). The specificity of the interaction between ERK2 and MKP-3 depends upon the latter having a kinase interaction motif (KIM) that binds to a specific site on ERK. This interaction enables the protein tyrosine phosphatase (PTP) domain to dephosphorylate the two phosphorylated residues on ERK2, thus curtailing its ability to stimulate downstream responses.

first described as a regulator of the cell cycle in studies on yeast cells, and still retains its yeast nomenclature. The three human isoforms also act to regulate the cell cycle by controlling both the entry into S phase (Cdc25A) and the entry into mitosis (Cdc25B and C) (Module 9: Figure cell cycle signalling mechanisms). The level of Cdc25A increases in late G1 and remains high throughout the rest of the cell cycle. The level of Cdc25B is increased during S phase to activate the entry into mitosis, and returns to a low level after mitosis is complete. The level of Cdc25C remains high throughout the cell cycle. All three isoforms have a similar C-terminal catalytic region, whereas the N-terminus, which has the regulatory regions, is somewhat variable. The activity of the Cdc25 isoforms is regulated by both activating and inhibitory phosphorylation. All three isoforms contain a phosphorylation site, which controls the binding of 14-3-3 protein that then inhibits the enzyme. This inhibitory site is phosphorylated by enzymes that are activated by cell stress, such as DNA damage. This stress-induced inhibition of the Cdc25 isoforms is thus an important mechanism for both G1 and G2/M cell cycle arrest.

The expression of Cdc25A is controlled by E2F. Once Cdc25A is expressed in the cytoplasm, it is available to activate cyclin-dependent kinase 2 (CDK2) to initiate the process of DNA synthesis. The activity of Cdc25A is very sensitive to DNA damage, which activates the checkpoint kinases 1 and 2 (CHK1 and CHK2) to phosphorylate Ser-123, which then promotes ubiquitination and rapid degradation. CHK1 is also responsible for phosphorylating Thr-507, which facilitates its interaction with 14-3-3 protein, which keeps the enzyme inactive until it is required.

Cdc25B, which plays an important role in the way cyclin B controls mitosis, is activated at the G2/M transition (Module 9: Figure mitotic entry). Like the other Cdc25 isoforms, Cdc25B is kept quiescent by phosphorylating Ser-323 that provides a binding site for 14-3-3 protein. This site is phosphorylated by the p38 pathway and provides a mechanism whereby this component of the mitogen-activated protein kinase (MAPK) signalling pathway can arrest the cell cycle (Module 2: Figure MAPK signalling).

The Cdc25C enzyme is kept quiescent through phosphorylation of Ser-216, which provides a binding site for 14-3-3 protein. During entry into mitosis, this inhibitory phosphate is removed and this enables the Polo-like kinases (Plks) to phosphorylate other sites in the regulatory region that enables Cdc25C to begin to dephosphorylate CDK1-activating kinases (Module 9: Figure cell cycle signalling mechanisms).

**Protein serine/threonine phosphatases**

There are a very large number of kinases that contribute to the ON reactions of cell signalling by phosphorylating both serine and threonine residues on target
**Module 5: Table serine/threonine phosphatase classification**

**Classification of the protein serine/threonine phosphatases**

<table>
<thead>
<tr>
<th>Phosphatase Comment</th>
<th>Comment</th>
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<tbody>
<tr>
<td><strong>PPM family</strong></td>
<td></td>
</tr>
<tr>
<td>PP1 (protein phosphatase 1)</td>
<td>There are three PP1 genes that give rise to four isoforms; PP1 has multiple regulatory components (Module 5: Table 5.1). PP1 regulatory, targeting and inhibitory subunits and proteins (Module 5: Table PP1 regulatory, targeting and inhibitory subunits and proteins). The activity of PP1 is inhibited by inhibitor 1 (I-1). The function of PP1 is determined by its associated proteins that regulate its activity and are responsible for targeting it to its specific sites of action. (Information reproduced and adapted from Cohen 2002.)</td>
</tr>
<tr>
<td>PP2A (protein phosphatase 2A)</td>
<td>An abundant and ubiquitous phosphatase that has multiple scaffolding and regulatory subunits (Module 5: Figure PP2A holoenzyme).</td>
</tr>
<tr>
<td>PP2B (calcineurin)</td>
<td>A Ca2+-sensitive protein phosphatase (Module 4: Figure calcineurin)</td>
</tr>
<tr>
<td>PP4 (protein phosphatase 4)</td>
<td>May function in nuclear factor κB (NF-κB) signaling and histone deacetylase 3 (HDAC3) dephosphorylation</td>
</tr>
<tr>
<td>PP5 (protein phosphatase 5)</td>
<td>May function in control of cell growth</td>
</tr>
<tr>
<td>PP6 (protein phosphatase 6)</td>
<td>May function in G2/S transition of cell cycle</td>
</tr>
<tr>
<td>PP7 (protein phosphatase 7)</td>
<td>Located in retinal and brain cells; the PPP family contains approximately nine human genes; little is known about most of these enzymes except for PP2C and Ppm2</td>
</tr>
<tr>
<td>PPM family</td>
<td>Prototypic member of the PPM family; implicated in dephosphorylation of cyclin-dependent kinases (CDKs), regulation of RNA splicing and control of p53 activity.</td>
</tr>
<tr>
<td>Ppm2</td>
<td>Pyruvate dehydrogenase phosphatase</td>
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</tbody>
</table>

The protein serine/threonine phosphatases are divided into two main families, the main phosphoprotein phosphatase (PPP) family and the smaller Mg2+-dependent protein phosphatase (PPM) family. By contrast, there is a relatively small group of protein serine/threonine phosphatases that remove these serine and threonine phosphates, thus reversing the activity of the kinases as part of the OFF reaction. The serine/threonine phosphatase classification reveals that most of these kinases belong to either the phosphoprotein phosphatase (PPP) family or the Mg2+-dependent protein phosphatase (PPM) family.

**Serine/threonine phosphatase classification**

There are two major families of serine/threonine phosphatases (Module 5: Table serine/threonine phosphatase classification). With regard to signalling, three members of the PPP family are particularly abundant and important with regard to cell signalling:

- **Protein phosphatase 1 (PP1)**
- **Protein phosphatase 2A (PP2A)**
- **Protein phosphatase 2B (PP2B)**

**Protein phosphatase 1 (PP1)**

Three genes code for the protein phosphatase 1 (PP1) catalytic subunit (PP1c), which give rise to four isoforms (PP1α, PP1β, PP1γ1 and PP1γ2). Despite this limited number of catalytic subunits, PP1 performs a large number of functions operating in many different cellular locations. It owes this versatility to the fact that it can interact with a large number of regulatory and inhibitory proteins. The activity of PP1 is inhibited by inhibitor 1 (I-1). The function of the PP1 regulatory/targeting and inhibitory

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**Module 5: Table PP1 regulatory, targeting and inhibitory subunits and proteins**

The regulatory, targeting and inhibitory subunits and proteins of protein phosphatase 1 (PP1).

<table>
<thead>
<tr>
<th>Subunit or protein</th>
<th>Cellular location and function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen targeting</td>
<td>Directs protein phosphatase 1 (PP1) to glycogen particles in skeletal and heart muscle; controls glycogen metabolism (Module 5: Figure PP1 targeting to glycogen)</td>
</tr>
<tr>
<td>GL</td>
<td>Directs PP1 to glycogen particles in liver; controls glycogen metabolism; distributed widely, but high in liver and muscle</td>
</tr>
<tr>
<td>RS</td>
<td>Also known as protein targeting to glycogen (PTG) (Module 6: Figure glycogen scaffold)</td>
</tr>
<tr>
<td>Myosin/actin targeting</td>
<td>Directs PP1 to myofibrils in smooth muscle cells and non-muscle cells; also known as myosin-binding subunit (MBS); controls smooth muscle relaxation (Module 7: Figure smooth muscle cell E-C coupling)</td>
</tr>
<tr>
<td>MYPT1 (myosin phosphatase targeting subunit)</td>
<td>Directs PP1 to myofibrils in skeletal muscle, where it controls contraction; also found in heart and brain</td>
</tr>
<tr>
<td>Neurabin I</td>
<td>Neuronal plasma membrane and actin cytoskeleton; functions in neurite outgrowth and synapse morphology</td>
</tr>
<tr>
<td>Spinophilin (Neurabin II)</td>
<td>Widespread location on plasma membrane and actin; attaches PP1 to ryanodine receptors (Module 3: Figure ryanodine receptor structure)</td>
</tr>
<tr>
<td>A-kinase-anchoring protein 220 (AKAP220)</td>
<td>Brain and testis, where it is located on cytoskeleton to co-ordinate protein kinase A (PKA) and PP1 signalling. Located in the neuronal postsynaptic density (Module 10: Figure postsynaptic density), where it modulates synaptic transmission</td>
</tr>
<tr>
<td>PP1 inhibitory proteins</td>
<td></td>
</tr>
<tr>
<td>I-1 (Inhibitor 1)</td>
<td>This inhibitor of PP1 is widely distributed</td>
</tr>
<tr>
<td>I-2 (Inhibitor 2)</td>
<td>This inhibitor of PP1 is found in brain and kidney</td>
</tr>
</tbody>
</table>
Module 5: Figure PP1 targeting to glycogen

Function of the regulatory proteins GM and GL in targeting protein phosphatase 1 (PP1) to both glycogen and the sarcoplasmic reticulum.

The regulatory protein GM in skeletal muscle or GL in liver has two targeting domains. One is located in the middle of the molecule (shown in green) that directs protein phosphatase 1 (PP1) to glycogen, where it can dephosphorylate the enzymes glycogen synthase and phosphorylase that control glycogen synthesis and glycogenolysis respectively. The other is a transmembrane region (shown in blue) in the C-terminal region that directs PP1 towards the sarcoplasmic reticulum, where it acts to dephosphorylate phospholamban, which functions to regulate the activity of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase 2 (SERCA2a) pump (Module 5: Figure phospholamban mode of action).

The regulatory subunits determine the substrate specificity and variable intracellular locations of PP1, which functions in the control of many cellular processes:

- The myosin phosphatase targeting subunit 1 (MYPT1) functions to localize PP1 to the myosin filaments in the contractile ring that controls cytokinesis during cell division (Module 9: Figure cytokinesis).
- PP1 plays an important role in the control of glycogen metabolism. In liver cells, GL targets PP1 to glycogen, where it functions to dephosphorylate glycogen synthase and phosphorylase (Module 7: Figure glycogenolysis and gluconeogenesis). The glycogen-targeting subunit GM in skeletal muscle also directs PP1 to the surface of glycogen granules, where it has a similar function. In skeletal muscle, it also targets PP1 to the sarcoplasmic reticulum (Module 5: Figure PP1 targeting to glycogen), where it functions to dephosphorylate the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump inhibitory phospholamban (Module 5: Figure phospholamban mode of action).
- PP1 controls smooth muscle relaxation (Module 7: Figure smooth muscle cell E-C coupling).
- Activity of the striatal-enriched phosphatase (STEP), which dephosphorylates ERK in neurons, is regulated by PP1 (Module 10: Figure medium spiny neuron signalling).
- PP1 contributes to the regulation of the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\)cotransporter 1 (NKCC1) (Module 3: Figure cation chloride cotransporter).
- Cell volume regulation in response to hypotonicity depends upon the dephosphorylation of the K\(^{+}\)-Cl\(^{-}\)cotransporter 1 (KCC1) by PP1 (Module 3: Figure cell volume regulation).
- The phosphorylation of AMPA receptors is regulated by PP1 and inhibitor 1 (I1) (Module 3: Figure AMPA receptor phosphorylation).

Dopamine and cyclic AMP-regulated phosphoprotein of apparent molecular mass 32 kDa (DARPP-32)

DARPP-32 is a dopamine and cyclic AMP-regulated phosphoprotein of apparent molecular mass 32 kDa, which functions as a molecular switch to regulate the activity of protein phosphatase 1 (PP1). As its name implies, it is regulated by protein kinase A (PKA)-dependent phosphorylation and is localized in dopamine-sensitive neurons such as the medium spiny neurons found in the dorsal striatum and nucleus accumbens. DARPP-32 may function as a node to co-ordinate the activity of the dopamine and glutamate signalling pathways (Module 10: Figure medium spiny neuron signalling). This integration of two separate neural signalling pathways may underlie the neural plasticity that occurs during drug addiction.

Protein phosphatase 2A (PP2A)

Protein phosphatase 2A (PP2A) is one of the most abundant of the serine/threonine protein phosphatases: it is
estimated to make up about 0.3% of total cellular protein. It is a highly versatile enzyme in that it can operate in many different cellular regions. This versatility depends upon the protein phosphatase 2A (PP2A) holoenzyme organization, which is a trimeric structure consisting of the scaffolding protein A subunit of protein phosphatase 2A, a regulatory B subunit and a catalytic C subunit (Module 5: Figure PP2A holoenzyme). There are a large number of regulatory B subunits, which are responsible for directing the holoenzyme to different cellular locations. Protein phosphatase 2A (PP2A) function depends primarily on its role in reversing the phosphorylation events that are part of many signalling pathways, and particularly those controlling processes such as development, differentiation, morphogenesis and cell proliferation. Its inhibitory role in cell proliferation has led to its classification as a tumour suppressor.

A mutation arising from expansion of a CAG trinucleotide repeat of the Bβ gene (Module 5: Table PP2A subunits) is the cause of autosomal dominant spinocerebellar ataxia type 12 (SCA12). A role for PP2A in cancer has emerged from the relationship between protein phosphatase 2A (PP2A) and tumour suppression.

**Protein phosphatase 2A (PP2A) holoenzyme organization**

Protein phosphatase 2A (PP2A) is a highly versatile enzyme that dephosphorylates a diverse array of proteins located in many different cellular locations. It owes this versatility to a large family of regulatory B proteins, which are part of the PP2A molecular toolkit (Module 5: Table PP2A subunits). The holoenzyme is a trimer composed of a PP2A scaffolding A subunit, which binds to a regulatory B subunit and a catalytic C subunit (Module 5: Figure PP2A holoenzyme). Given that there are two A subunits, two C subunits and at least 13 B subunits, many combinations are possible, resulting in multiple heterotrimeric holoenzymes. Much of the versatility of this enzyme depends on the large number of B regulatory subunits that have subtly different properties, especially with regard to their ability to direct holoenzymes to different cellular regions and substrates. Some of the roles of the B subunit in determining PP2A function are summarized in Module 5: Table PP2A subunits, but many of the targeting functions are still being elucidated.

**Protein phosphatase 2A (PP2A) function**

The primary role of protein phosphatase 2A (PP2A) is to dephosphorylate many of the phosphoproteins that function in cell signalling pathways:

- PP2A can modulate the mitogen-activated protein kinase (MAPK) signalling pathway both positively and negatively. With regard to the former, it can dephosphorylate some of the inhibitory sites on Raf-1. In addition, it can inhibit the signalling cascade by reversing some of the phosphorylation events downstream of Raf-1 (Module 2: Figure ERK signalling).
- Some of the key phosphorylation events of the canonical Wnt/β-catenin pathway are reversed by PP2A (Module 2: Figure Wnt canonical pathway).
- Protein kinase A (PKA)-dependent phosphorylation of the L-type CaV1.2 channel is reversed by PP2A (Module 3: Figure CaV1.2 L-type channel).
- PKA-dependent phosphorylation of the type 2 ryanodine receptor (RYR2) in cardiac cells is reversed by PP2A (Module 3: Figure ryanodine receptor structure).
- PP2A interacts with the scaffolding protein A-kinase-anchoring protein 350 (AKAP350) localized on the centrosome; PR130 links PP2A to the ryanodine receptor (Module 3: Figure ryanodine receptor structure).
- A large number of genes are used to encode the scaffolding, regulatory and catalytic subunits that are used to make up the diverse array of protein phosphatase 2A (PP2A) holoenzymes (Module 5: Figure PP2A holoenzyme).

### Table PP2A subunits Cellular location and function

<table>
<thead>
<tr>
<th>PP2A subunits</th>
<th>Cellular location and function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP2A scaffolding A subunits</td>
<td>Neuronal cell bodies and nucleus; linked to microtubules and is a tau phosphatase</td>
</tr>
<tr>
<td>PP2A regulatory B subunits</td>
<td>Abundant in brain and testes; in brain, it is in the cell body (excluding the nucleus) and extends into axons and dendrites; linked to microtubules and is a tau phosphatase; mutated in spinocerebellar ataxia type 12 (SCA12)</td>
</tr>
<tr>
<td>PR130</td>
<td>Abundant in brain and testes</td>
</tr>
<tr>
<td>PR59</td>
<td>Skeletal muscle and cardiac cells; targets PP2A to the apoptotic protein Bcl-2</td>
</tr>
<tr>
<td>PR72</td>
<td>Brain</td>
</tr>
<tr>
<td>PR73</td>
<td>Brain and testis</td>
</tr>
<tr>
<td>PR98</td>
<td>Located in the nucleus, where it interacts with Cdc6 in the pre-replication complexes during DNA synthesis</td>
</tr>
<tr>
<td>PR73</td>
<td>Interacts with p105, a retinoblastoma (Rb)-related protein that can arrest the cell cycle by dephosphorylating the transcription factor E2F</td>
</tr>
<tr>
<td>PR48</td>
<td>Directs PP2A to the signalling complex assembled on A-kinase-anchoring protein 350 (AKAP350) localized on the centrosome; PR130 links PP2A to the ryanodine receptor (Module 3: Figure ryanodine receptor structure)</td>
</tr>
</tbody>
</table>

A large number of genes are used to encode the scaffolding, regulatory and catalytic subunits that are used to make up the diverse array of protein phosphatase 2A (PP2A) holoenzymes (Module 5: Figure PP2A holoenzyme).
Assembly of the protein phosphatase 2A (PP2A) holoenzyme.
The protein phosphatase 2A (PP2A) holoenzyme is assembled from three subunits that have different functions. The molecular framework is provided by the scaffolding subunit (A), which is made up of 15 non-identical repeats, which are organized into a hook-shaped molecule. These repeats are connected by inter-repeat loops (shown in blue). Each repeat has two α-helices that are connected by intra-repeat loops (shown in orange), which line up to provide a cradle to bind the other subunits. Loops 1–10 are responsible for binding one of the regulatory B subunits, which belong to three families (B, B’ and B’’). There are two PP2A catalytic subunits (Cα and Cβ) and one of these attaches to loops 11–15. This recruitment of the catalytic subunit into the holoenzyme depends upon carboxymethylation of Leu-309 by leucine carboxmethyltransferase (LCMT) and is reversed by a phosphatase methylesterase (PME-1). Once assembled, the holoenzyme functions to dephosphorylate a wide range of phosphorylated substrates.

α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor (Module 10: Figure postsynaptic density).
• The phosphorylation status of the neuron-specific microtubule-associated protein tau, which has been implicated in Alzheimer’s disease, is regulated by the PP2A holoenzyme carrying the Bβ regulatory subunit (Module 5: Table PP2A subunits).
• PP2A functions in Myc degradation.

Protein phosphatase 2A (PP2A) and tumour suppression
One of the important actions of protein phosphatase 2A (PP2A) is to regulate cell proliferation, where it normally acts to reverse the protein phosphorylation of the proliferation signalling pathways driven by various growth factors (Module 9: Figure proliferation signalling network). For example, PP2A contributes to Myc degradation, which is an important regulator of cell proliferation and is often amplified in many human cancers. Modifications of PP2A either through mutation of its subunits or by interactions with viral proteins can cause cancer (Module 5: Figure PP2A modifications and cancer). The negative effects on cell growth have led to the concept of PP2A functioning as a tumour suppressor. Some of the most convincing evidence for this comes from the finding that simian virus 40 (SV40) small T antigen and polyoma virus small T and middle T antigens bind to the scaffolding A subunit, resulting in a decrease in phosphatase activity.

Protein phosphatase 2B (PP2B)
Protein phosphatase 2B (PP2B) is known more commonly as calcineurin (CaN), which is a Ca2+-activated serine/threonine phosphatase (Module 4: Figure calcineurin).

Protein phosphatase 4 (PP4)
Not much is known about protein phosphatase 4 (PP4). Like the other serine/threonine phosphatases, PP4 is made up of a catalytic subunit (PP4C) that interacts with various regulatory subunits (R1, R2, R3 and α4). In addition, it can interact with signalling proteins such as nuclear factor κB (NF-κB) and histone deacetylase 3 (HDAC3). There is increasing evidence that PP4 may have a highly specific role in modulating a variety of signalling mechanisms. For example, it can activate NF-κB by dephosphorylating Thr-43. It may play a role in histone acetylation and chromatin remodelling by dephosphorylating HDAC3.

Phosphodiesterase (PDE)
The OFF mechanism of the cyclic AMP signalling pathway and the cyclic GMP signalling pathway is carried out by phosphodiesterases (PDEs) that inactivate the two cyclic nucleotide second messengers (cyclic AMP and
Modifications of protein phosphatase 2A (PP2A) by mutations and interactions with viral proteins can cause cancer. PP2A is considered to be a tumour suppressor because cancers can develop or be exacerbated when the activity of this enzyme is reduced either by mutations of the subunits or by interactions with viral proteins:

1. Mutations of the scaffolding A subunit, which then fails to bind the B and C subunits, have been identified in a number of human cancers (breast, colon, lung and skin).
2. Truncation of the B subunit, which prevents it from interacting with the catalytic C subunit, has been implicated in metastasis.
3. Tumour-promoting viruses act by binding to the scaffolding A subunit to displace the regulatory B subunit.

PDE1

The characteristic feature of PDE1 is that it is activated by \( \text{Ca}^{2+} \). This \( \text{Ca}^{2+} \) sensitivity depends on the \( \text{Ca}^{2+} \) sensor calmodulin (CaM) which binds to two CaM-binding domains located in the regulatory N-terminal region of PDE1 (Module 5: Figure PDE domains). The PDE1 family consists of three genes.

**PDE1A**

PDE1A, which has five splice variants, has a higher affinity for cyclic GMP (\( K_m \) approximately 5 \( \mu \)M) than cyclic AMP (\( K_m \) approximately 110 \( \mu \)M). Phosphorylation of PDE1A1 and PDE1A2 by protein kinase A (PKA) results in a decrease in its sensitivity to \( \text{Ca}^{2+} \) activation.

**PDE1B**

PDE1B, which has two splice variants, has a higher affinity for cyclic GMP (\( K_m \) approximately 2.7 \( \mu \)M) than for cyclic AMP (\( K_m \) approximately 24 \( \mu \)M). This isoform is strongly expressed in the brain. Phosphorylation of PDE1B by \( \text{Ca}^{2+}/\text{calmodulin-dependent protein kinase II (CaMKII)} \) results in a decrease in its sensitivity to \( \text{Ca}^{2+} \) activation.

**PDE1C**

PDE1C, which has five splice variants, has a high affinity for both cyclic GMP and cyclic AMP (\( K_m \) approximately 1 \( \mu \)M). The PDE1C2 splice variant is located in olfactory sensory cilia, where it functions to regulate the role of cyclic GMP.

The PDEs belong to a large family comprising 11 PDE gene families (Module 5: Table PDE family properties). This extensive PDE family share one thing in common: they all hydrolyse cyclic nucleotide second messengers, but in other respects, they are very different with regard to substate specificity, kinetic properties, regulation and cellular distribution. Much of this variability resides in the N-terminal region, which has different domains that determine the unique characteristics of each family member (Module 5: Figure PDE domains). In the light of this enormous family diversity, it is difficult to make too many generalizations, so each family member is considered separately. Most information is available for PDE1–PDE6:

- **PDE1** is a \( \text{Ca}^{2+} \)-sensitive cyclic AMP phosphodiesterase.
- **PDE2** is a cyclic GMP-stimulated cyclic AMP phosphodiesterase.
- **PDE3** is a cyclic GMP-inhibited cyclic AMP phosphodiesterase.
- **PDE4** is a cyclic AMP phosphodiesterase.
- **PDE5** is a cyclic GMP-specific phosphodiesterase sensitive to Viagra.
- **PDE6** is the cyclic GMP phosphodiesterase in photoreceptors.

PDEs belong to a large family comprising 11 PDE gene families (Module 5: Table PDE family properties). This extensive PDE family share one thing in common: they all hydrolyse cyclic nucleotide second messengers, but in other respects, they are very different with regard to substate specificity, kinetic properties, regulation and cellular distribution. Much of this variability resides in the N-terminal region, which has different domains that determine the unique characteristics of each family member (Module 5: Figure PDE domains). In the light of this enormous family diversity, it is difficult to make too many generalizations, so each family member is considered separately. Most information is available for PDE1–PDE6:
Module 5: Table PDE family properties

Summary of the organization and properties of the 11 phosphodiesterase (PDE) families.

<table>
<thead>
<tr>
<th>PDE family</th>
<th>Gene</th>
<th>Number of splice variants</th>
<th>Regulatory domain, role</th>
<th>Phosphorylation</th>
<th>Substrate(s)</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE1</td>
<td>1A, 1B, 1C</td>
<td>9</td>
<td>CaM, activation</td>
<td>PKA</td>
<td>cGMP, cAMP</td>
<td>KS-505</td>
</tr>
<tr>
<td>PDE2</td>
<td>2A</td>
<td>3</td>
<td>GAF, activation</td>
<td>Unknown</td>
<td>cAMP, cGMP</td>
<td>EHNA</td>
</tr>
<tr>
<td>PDE3</td>
<td>3A, 3B</td>
<td>1 each</td>
<td>Transmembrane domains, membrane targeting</td>
<td>PKB</td>
<td>cAMP</td>
<td>Mirinone</td>
</tr>
<tr>
<td>PDE4</td>
<td>4A, 4B, 4C, 4D</td>
<td>&gt;20</td>
<td>UCR1, UCR2, unclear</td>
<td>ERK, PKA</td>
<td>cAMP</td>
<td>Rolipram</td>
</tr>
<tr>
<td>PDE5</td>
<td>5A</td>
<td>3</td>
<td>GAF, unclear</td>
<td>PKA, PKG</td>
<td>cGMP</td>
<td>Sildenafil, Dipyramidol, Zaprinast</td>
</tr>
<tr>
<td>PDE6</td>
<td>6A, 6B, 6C</td>
<td>1 each</td>
<td>GAF, activation</td>
<td>PKC, PKA</td>
<td>cGMP</td>
<td>Dipyramidol, Zaprinast</td>
</tr>
<tr>
<td>PDE7</td>
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<td>6</td>
<td>Unknown</td>
<td>Unknown</td>
<td>cAMP</td>
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<tr>
<td>PDE8</td>
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<td>6</td>
<td>PAS, unknown</td>
<td>Unknown</td>
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<tr>
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<td>Unknown</td>
<td>cGMP</td>
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<tr>
<td>PDE10</td>
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<td>2</td>
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<td>Unknown</td>
<td>cAMP, cGMP</td>
<td>None identified</td>
</tr>
<tr>
<td>PDE11</td>
<td>11A</td>
<td>4</td>
<td>GAF, unknown</td>
<td>Unknown</td>
<td>cAMP, cGMP</td>
<td>None identified</td>
</tr>
</tbody>
</table>

Some of the phosphodiesterase (PDE) families have more than one gene, and complexity is enhanced further by numerous splice variants. The different PDEs have variable substrate specificities: some hydrolyse either cyclic AMP or cyclic GMP, whereas others have dual specificity. Reproduced from *Handbook of Cell Signaling*, Vol. 2, Glick, J.L. and Beavo, J.A., Phosphodiesterase families, pp. 431–435. Copyright (2003), with permission from Elsevier; see Glick and Beavo 2003.

Module 5: Figure PDE domains

Domain structure of the phosphodiesterase (PDE) family that functions to hydrolyse and inactivate the second messenger cyclic AMP. Many of the phosphodiesterase (PDE) family members are characterized by having paired regulatory domains in the N-terminal regulatory region. PDE1 has Ca\(^{2+}\)/calmodulin-binding domains; PDE2, PDE5, PDE6, PDE10 and PDE11 have cyclic GMP-binding GAF domains; PDE4 has upstream conserved regulatory regions 1 and 2 (UCR1 and UCR2). Reproduced from *Handbook of Cell Signaling*, Vol. 2, Glick, J.L. and Beavo, J.A., Phosphodiesterase families, pp. 431–435. Copyright (2003), with permission from Elsevier; see Glick and Beavo 2003.

cyclic AMP in transducing odorant stimuli (Module 10: Figure olfaction) It is also expressed in β-cells, where it functions to regulate glucose-induced insulin secretion.

**PDE2**

PDE2 is a cyclic AMP phosphodiesterase that can be stimulated by cyclic GMP. PDE2 exists as a single gene (PDE2A) that has three splice variants that determine its subcellular distribution, with PDE2A1 being soluble, whereas PDE2A2 and PDE2A3 are particulate. The membrane location of PDE2A2 may depend upon a transmembrane segment in the N-terminal region, whereas PDE2A3 appears to associate with membranes through an N-terminal myristoylation site.

PDE2 is strongly expressed in the brain and is also found in skeletal muscle, heart, liver, adrenal glomerulosa and pancreatic cells.

Although PDE2 is a dual-specificity enzyme capable of hydrolysing both cyclic AMP and cyclic GMP, the enzyme seems to favour cyclic AMP because cyclic GMP acts as an allosteric regulator that greatly enhances the ability of PDE2 to hydrolyse cyclic AMP. It is for this reason that this enzyme is referred to as a cyclic GMP-stimulated cyclic AMP PDE.

The ability of cyclic GMP to enhance the hydrolysis of cyclic AMP may account for the signalling cross-talk that occurs in some cells. For example, the nitric oxide (NO)/cyclic GMP-induced reduction in L-type Ca\(^{2+}\)}
channel activity in cardiac cells may depend upon cyclic GMP stimulating PDE2, thereby reducing the level of cyclic AMP that normally regulates these channels. Another example is found in zona glomerulosa cells, where atrial natriuretic factor (ANF) may inhibit the secretion of aldosterone by using cyclic GMP to increase the activity of PDE2 to reduce the level of cyclic AMP, which drives the release of this steroid (Module 7: Figure glomerulosa cell signalling).

**PDE3**

There are two genes encoding PDE3, which is a cyclic GMP-inhibited cyclic AMP phosphodiesterase. They are characterized by having six putative transmembrane segments in the N-terminal region (Module 5: Figure PDE domains), which seem to be responsible for targeting this enzyme to cell membranes. These two family members (PDE3A and PDE3B) have different functions and cellular locations.

**PDE3A**
PDE3A is located in blood platelets, smooth muscle cells and cardiac myocytes.

**PDE3B**
PDE3B is found in brown and white fat cells, pancreatic β-cells and liver cells, all of which are cells that function in energy metabolism. This isoform is particularly important as an effector for the action of insulin in antagonizing the catecholamine-dependent lipolysis and release of fatty acids from white fat cells (Module 7: Figure lipolysis and lipogenesis). Insulin acts through the PtdIns 3-kinase signalling pathway to increase the enzymatic activity of PDE3B, and the resulting decline in the activity of cyclic AMP leads to a decrease in lipid hydrolysis. A similar mechanism operates in liver cells to carry out the anti-glycogenolytic action of insulin (Module 7: Figure liver cell signalling). Insulin-like growth factor I (IGF-I) and leptin may reduce insulin secretion in response to GLP-1 by stimulating the activity of phosphodiesterase PDE3B, thereby reducing the level of cyclic AMP (Module 7: Figure β-cell signalling).

Insulin resistance and obesity may arise from a reduced expression of PDE3B.

**PDE4**
PDE4 functions only to hydrolyse cyclic AMP. It consists of four genes with approximately 20 splice variants, which fall into three main categories: long, short and super-short. Much of this variation depends upon the expression of their characteristic upstream conserved regions 1 and 2 (UCR1 and UCR2) in the N-terminal regulatory region (Module 5: Figure PDE domains). The long isoforms have both UCR1 and UCR2, the short isoforms lack UCR1, whereas the super-short isoforms lack UCR1 and have a truncated UCR2.

The activity of the various PDE4 isoforms can be regulated through a feedback loop operated through protein kinase A (PKA) and by inputs from other signalling pathways, such as the mitogen-activated protein kinase (MAPK) signalling pathway. The ability of PKA to modulate the activity of PDE4 is facilitated by the fact that they are both associated on the same scaffolding protein muscle A-kinase-anchoring protein (mAKAP).

**PDE4A**
PDE4A is located in the soma of olfactory neurons, in contrast with PDE1C2, which is in the cilium. PDE4A1 associates with membranes through a hydrophobic domain in the N-terminal region, whereas PDE4A5 is located at the plasma membrane, where it associates with proteins containing SH3 domains.

**PDE4B**
PDE4B plays an important role in inflammatory responses, because PDE4B−/− mice display a large decrease in their ability to release tumour necrosis factor α (TNFα) in response to lipopolysaccharide (LPS). The cyclic AMP signalling pathway functions in the modulation of inflammatory responses. It has an anti-inflammatory role in macrophages, and this inhibitory effect is usually dampened by the up-regulation of PDE4B (Module 11: Figure macrophage signalling). PDE4B has an important role in regulating the contractile activity of uterine smooth muscle cells.

**PDE4D**
PDE4D may play some role in asthma. PDE4D−/− mice have been found to lack normal muscarinic responses, resulting in a loss of airway hyperreactivity.

**PDE5**
There is a single cyclic GMP-specific phosphodiesterase (PDE5) gene with three splice variants. It is a cyclic GMP-specific phosphodiesterase, which has a unique feature in that it is also regulated by cyclic GMP binding to the tandem GAF domains in the regulatory region (Module 5: Figure PDE domains). Binding of cyclic GMP to these GAF domains is necessary for protein kinase A (PKA) or cyclic GMP-dependent protein kinase (cGK) to phosphorylate a single site in the N-terminal region, which then results in an increase in both the rate of catalysis and cyclic GMP-binding affinity of the catalytic site. This complex combination of regulation through both the allosteric binding of cyclic GMP and phosphorylation by cGK can result in different functional states of the enzyme (Module 5: Figure PDE5 functional states).

PDE5 plays a major role in regulating the cyclic GMP signalling pathway in various cells, such as smooth muscle cells (Module 7: Figure smooth muscle cell cGMP signalling), blood platelets, renal tissue (proximal and collecting ducts), cerebellar Purkinje cells and pancreatic ducts.

In the case of the corpus cavernosum smooth muscle cells, which regulate penile erection (Module 7: Figure corpus cavernosum), PDE5 is the target for Viagra, a drug used to treat male erectile dysfunction.
Module 5: Figure PDE5 functional states

A model depicting different functional states of PDE5.

PDE5 functions as a dimer with the two subunits connected through a region that includes the allosteric cyclic GMP-binding GAF domains. During the course of enzyme activation, cyclic GMP appears to bind first to the catalytic site ($K_m$ of 1–6 µM), which induces a conformational change that then enhances the affinity of cyclic GMP binding to the GAF domains. This binding to the regulatory region induces a further conformational change to expose the serine residue, which is then phosphorylated by cyclic GMP-dependent protein kinase (cGK). This phosphorylated state is the most active form of the enzyme. This thus represents a complex feedback loop whereby cyclic GMP promotes its own hydrolysis by binding allosterically to the enzyme and by promoting its phosphorylation by stimulating cGK. Reproduced from Handbook of Cell Signaling, Vol. 2, Francis, S.H. and Corbin, J.D., Phosphodiesterase-5, pp. 447–451. Copyright (2003), with permission from Elsevier; see Francis and Corbin 2003.

PDE6

PDE6 is a highly specialized enzyme that is the primary effector of visual transduction in vertebrate photoreceptors (Module 10: Figure phototransduction overview).

The stability of PDE6 is regulated by aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1). Just how AIPL1 functions is not entirely clear, but it appears to function as a specific chaperone required for PDE6 biosynthesis and stability. Leber congenital amaurosis (LCA), which is an early onset human retinopathy, has been linked to mutations in the $AIPL1$ gene.

Ca$^{2+}$ pumps and exchangers

A variety of pumps and exchangers are responsible for removing Ca$^{2+}$ from the cytoplasm (Module 5: Figure Ca$^{2+}$ uptake and extrusion). The most obvious function of such pumps is therefore to enable cells to recover from Ca$^{2+}$-induced signalling events. However, such pumps have two other important functions. Firstly, they ensure that the internal stores are kept loaded with signal Ca$^{2+}$ by pumping Ca$^{2+}$ into the sarcoplasmic reticulum (SR) of muscle cells or the endoplasmic reticulum (ER) of non-muscle cells. Pumps are also important for loading Ca$^{2+}$ into the Golgi. Secondly, they maintain the resting level of Ca$^{2+}$. The constant leakage of Ca$^{2+}$ into the cell down the very large concentration gradients facing the cytoplasm, both from the outside and from the internal stores, is expelled by pumps to ensure that the resting Ca$^{2+}$ concentration is held constant at approximately 100 nM. A pump classification reveals that there are five different mechanisms responsible for carrying out these functions of recovery, maintaining the Ca$^{2+}$ stores and the resting level of Ca$^{2+}$:

- Plasma membrane Ca$^{2+}$-ATPase (PMCA)
- Sodium/calcium exchangers (NCX and NCKX)
- Sarco/endo-plasmic reticulum Ca$^{2+}$-ATPase (SERCA)
- Mitochondrial uniporter
- Secretory-pathway Ca$^{2+}$-ATPase (SPCA)

Two of these pumps (PMCA and NCX) are located on the plasma membrane, whereas the others are located on internal organelles. The organization and distribution of Ca$^{2+}$ pumps determines the properties of Ca$^{2+}$ pumps, which are adapted to carry out different homeostatic functions. The PMCA pump family consists of four genes with diversity enhanced by alternative splicing at two sites. The SERCA family has three genes, and alternative splicing gives at least six different isoforms. Likewise, the NCX has a family of three genes, and alternative splicing gives rise to numerous isoforms. The way in which alternative splicing can enhance diversity is illustrated for SERCA2a and its isoforms, which have not only different properties, but also different distributions. In summary, the molecular organization gives rise to a diverse repertoire of pumps from which cells can select that combination of pumps that exactly meets their Ca$^{2+}$ signalling requirements.

The molecular structure of the Ca$^{2+}$ pumps is designed to transfer Ca$^{2+}$ ions across membranes against very large electrochemical gradients. The exception to this is the mitochondrial uniporter, which is not a pump in the strict sense, but it is a channel that allows Ca$^{2+}$ to flow from the cytoplasm into the mitochondrial matrix. The plasma membrane Ca$^{2+}$-ATPase (PMCA) molecular structure and that of the SERCA pump are very similar with regard to their main domains. They have ten transmembrane domains, with both the N-terminal and C-terminal ends
facing the cytoplasm. The NCX and NCKX molecular structure consists of nine and 11 transmembrane domains respectively. They both have a large cytoplasmic loop connecting transmembrane domains 5 and 6 (Module 5: Figure sodium/calcium exchangers).

The different structural domains have specific functions, which have been well described for the sarco/endo-plasmic reticulum Ca2+-ATPase (SERCA) pump structure and mechanism. There is less information on the NCX pump mechanism, where the energy to pump Ca2+ is derived from the flow of Na+ down its electrochemical gradient.

Ca2+ pump regulation plays a critical role in enabling pumps to deal with large variations in the intracellular level of Ca2+.

Alterations in the way cells pump Ca2+ have been linked to a variety of diseases. For example, Darier’s disease is an autosomal skin disorder that results from a loss of one copy of the SERCA2 gene. Brody disease results from a defect in the SERCA1a pump that is responsible for relaxing skeletal muscle. Hailey-Hailey disease is caused by an inactivating mutation of the secretory Ca2+ pump.

Properties of Ca2+ pumps
The different Ca2+ pumping mechanisms have very different properties with regard to their affinity for Ca2+ and the rate at which they can transport this ion across membranes (Module 5: Figure Ca2+ uptake and extrusion):

Low-affinity, high-capacity pumps
The Na+/Ca2+ exchanger (NCX), the Na+/Ca2+/K+ exchanger (NCKX) and the mitochondrial uniporter, for example, have low affinities for Ca2+, but have very high capacities, and this enables them to function early in the recovery process, since they can rapidly remove the large quantities of Ca2+ that are released into the cytoplasm during signalling. The high capacity of NCX and NCKX is based on the rapid turnover rate of the exchanger, which can carry out 1000 to 5000 reactions/s.

High-affinity, low-capacity pumps
On the other hand, the plasma membrane Ca2+-ATPase (PMCA), sarco/endo-plasmic reticulum Ca2+-ATPase (SERCA) and secretory-pathway Ca2+-ATPase (SPCA) pumps have lower capacities, but their higher affinities mean that they can continue to pump at lower Ca2+ levels, thus enabling them to maintain the internal stores and the resting level. The SPCA is unusual in that it can pump Mn2+ equally as well as Ca2+. The PMCA and SERCA pumps have low capacities, because the ATP-dependent conformational process that occurs during the pumping mechanism occurs at a low rate (approximately 150 reactions/s). These two pumps belong to the P2 subfamily of P-type ion transport ATPases that are characterized by the formation of an aspartyl phosphate during the reaction cycle of the pump mechanism.

Organization and distribution of Ca2+ pumps
Ca2+ pumps have molecular structures designed to transfer Ca2+ ions across membranes against very large electrochemical gradients. This pumping problem has been solved in different ways. The diversity of Ca2+ pumps depends upon the existence of multigene families (Module 5: Table Ca2+ pumping toolkit) within which additional diversity is generated by alternative splicing. This diversity creates many isoforms with subtle variations, not only in their pumping properties but also in their Ca2+ pump regulation. An important consequence of all this diversity is that each cell has access to an enormous repertoire from which it can select out those pumps with properties exactly suited to their particular signalling requirements.

Plasma membrane Ca2+-ATPase (PMCA)
The plasma membrane Ca2+-ATPase (PMCA) gene family contains four closely related genes (PMCA1–PMCA4)
**Module 5: **Figure Ca\(^{2+}\) uptake and extrusion

![Diagram of Ca\(^{2+}\) uptake and extrusion](image)

**Functional organization of Ca\(^{2+}\) pumps.**

The Na\(^+/\)Ca\(^{2+}\) exchanger (NCX) and the mitochondrial uniporter are particularly effective at pumping Ca\(^{2+}\) when the cytosolic Ca\(^{2+}\) concentration is high, in that they combine a low affinity for Ca\(^{2+}\) with a high capacity. The plasma membrane Ca\(^{2+}\)-ATPase (PMCA) and sarco/endo-plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pumps have much lower capacities, but their much higher affinities enable them to reduce the level of Ca\(^{2+}\) back to its resting level. The different extrusion mechanisms thus co-operate with each other to regulate the level of Ca\(^{2+}\) over a large dynamic range.

with numerous alternatively spliced forms denoted by the lower-case letters of the alphabet (Module 5: Table Ca\(^{2+}\) pumping toolkit). Expression of these various splice isoforms is a regulated event in that they change in a consistent way during both development and differentiation. There are indications that changes in the level of Ca\(^{2+}\) may influence the expression of these splice isoforms. For example, an elevation in the level of Ca\(^{2+}\) in cerebellar granule cells results in the up-regulation of PMCA1a, PMCA2 and PMCA3, but a down-regulation of PMCA4a. The main domain structure of the PMCA is the presence of ten transmembrane (TM) domains with two large cytosolic loops between TM2 and TM3 and between TM4 and TM5 (Module 5: Figure PMCA domain structure). The latter is particularly significant, because it contains the aspartyl phosphorylation site (P). These two loops have important functions in Ca\(^{2+}\) pump regulation of PMCA activity. The PMCA isoforms 1 and 4 are widely expressed (Module 5: Table Ca\(^{2+}\) pumping toolkit), whereas isoforms 2 and 3 are mainly restricted to the brain and skeletal muscle. Within the brain, there are regional differences in the expression of these isoforms, e.g. PMCA2 is high in cerebellar Purkinje cells and cochlear hair cells, whereas PMCA3 is found mainly in the choroid plexus.

One consequence of pump diversity is that cells have access to pumps that transport at different rates. Cells that have to generate rapid transients have the fastest pumps. For example, PMCA3f (skeletal muscle) and PMCA2a (stereocilia) are the fastest, whereas PMCA4b (Jurkat cells) is the slowest.

In kidney tubule cells, the PMCA1b plays an important role in the reabsorption of Ca\(^{2+}\) by the paracellular transport pathway (Module 7: Figure kidney Ca\(^{2+}\) reabsorption).

**Plasma membrane Ca\(^{2+}\)-ATPase (PMCA) molecular structure**

Despite the large molecular diversity within the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) family, the overall structure of all the members is very similar. They have ten transmembrane domains with both the N-terminal and C-terminal regions facing the cytosol (Module 5: Figure PMCA domain structure). Most of the extracellular and intracellular loops that link the transmembrane domains are relatively short, except for two of the four loops that face the cytosol. The largest cytoplasmic loop connecting TM4 and TM5 is of particular significance because it contains two important sites for the pump cycle. The first site is the nucleotide-binding domain, where the ATP binds to the pump molecule. The second site is the phosphorylation site, which contains the invariant aspartate residue that is phosphorylated during the conformational changes that occur during each pump cycle (Module 5: Figure SERCA pump cycle).

The location of the PMCA pumps may be determined by binding to a family of PDZ domain-containing
Domain structure of the plasma membrane Ca\(^{2+}\)-ATPase (PMCA).

A. The sites marked A and C are the main sites where alternative splicing occurs to create at least 20 different isoforms. These two splice sites occur in the two large cytoplasmic loops, and are thus likely to influence the way in which these two loops regulate pump activity. B. In the absence of calmodulin (CaM), the autoinhibitory C-terminal region is thought to bend around to inhibit enzymatic activity. In the presence of Ca\(^{2+}\), CaM binds to the CaM-binding domain (CaMBD), and this regulatory chain is pulled away, resulting in an increase in pump activity. Reproduced from Strehler, E.E. and Zacharias, D.A. (2001) Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. Physiol. Rev. 81:21–50; used with permission from The American Physiological Society; see Strehler and Zacharias 2001.

proteins. Such an interaction may occur through the PDZ interaction domains located in the C-terminal region.

Sarco/endo-plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)

The sarco/endo-plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) family of pumps contains three genes with numerous alternatively spliced isoforms (Module 5: Table Ca\(^{2+}\) pumping toolkit). The role of SERCA is to pump Ca\(^{2+}\) back into the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) (Module 5: Figure Ca\(^{2+}\) uptake and extrusion). There have been considerable advances in the understanding of the sarco/endo-plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump structure and mechanism of Ca\(^{2+}\) transfer across the ER/SR membrane.

Inactivating mutations of SERCA1 are the cause of Brody disease. A mutation of the SERCA2 pump causes Darier’s disease.

Sarco/endo-plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump structure and mechanism

The resolution of the sarco/endo-plasmic Ca\(^{2+}\)-ATPase (SERCA) pump structure has provided a detailed scenario of how this pump might work. For the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) and SERCA pumps, some of the transmembrane domains make up the Ca\(^{2+}\)-binding site used to transfer Ca\(^{2+}\) across the membrane. ATP provides the energy to drive this transfer. Like the PMCA pump, the SERCA pump is a member of the P-type pumps, so called because the pump is energized by ATP phosphorylating an aspartate residue. This phosphorylation event induces the conformational change necessary to drive Ca\(^{2+}\) across the membrane. The following sequence of events occurs during the SERCA pump cycle (Module 5: Figure SERCA pump cycle):

1. The resting E\(_1\) state is energized by binding ATP to unveil two Ca\(^{2+}\)-binding sites that face the cytoplasm (E\(_1\).ATP).
2. Ca\(^{2+}\) enters the two binding sites to form the E\(_1\).ATP.2Ca\(^{2+}\) complex.
3. The binding of Ca\(^{2+}\) strongly activates the ATPase activity of the pump, resulting in the release of ADP and the transfer of phosphate to an aspartate residue to form a high-energy phosphorylated intermediate (E\(_1\)–P~2Ca\(^{2+}\)).
4. The energy stored in this phosphorylated intermediate is used to induce the conformational change to the E\(_2\)–P2Ca\(^{2+}\) state during which Ca\(^{2+}\) moves across the bilayer.
5. In the lower-energy E\(_2\)–P2Ca\(^{2+}\) state, the binding sites have a reduced affinity for Ca\(^{2+}\), which is free to diffuse into the lumen.
6. Hydrolysis of the E\(_2\)–P phosphoenzyme enables the pump to return to the resting E\(_1\) state, ready to begin another cycle.

The next question to consider is the molecular basis of this pump cycle. How are the individual steps of the pump cycle (Module 5: Figure SERCA pump cycle) related to the molecular structure of SERCA? A feature of SERCA structure is characterized by a number of distinct domains (Module 5: Figure SERCA1a pump), which have clearly defined roles at different stages of the pump cycle.
Module 5: Table Ca^{2+} pumping toolkit

Summary of genomic organization, spliced isoform and distribution of Ca^{2+} pumps and exchangers.

<table>
<thead>
<tr>
<th>Component</th>
<th>Spliced isoform</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma membrane Ca^{2+}-ATPase (PMCA) pumps</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMCA1 (the human gene is located on 12q21−q23)</td>
<td>PMCA1a</td>
<td>Excitable cells: brain, skeletal muscle, heart and kidney</td>
</tr>
<tr>
<td></td>
<td>PMCA1b</td>
<td>Ubiquitous; a housekeeper pump</td>
</tr>
<tr>
<td></td>
<td>PMCA1c</td>
<td>Skeletal muscle, heart</td>
</tr>
<tr>
<td></td>
<td>PMCA1d</td>
<td>Skeletal muscle, heart</td>
</tr>
<tr>
<td></td>
<td>PMCA1e</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>PMCA1x</td>
<td>Ubiquitous; a housekeeper pump</td>
</tr>
<tr>
<td>PMCA2 (the human gene is located on 3p25−p26)</td>
<td>PMCA2a</td>
<td>Brain, heart, uterus</td>
</tr>
<tr>
<td></td>
<td>PMCA2b</td>
<td>Widespread</td>
</tr>
<tr>
<td></td>
<td>PMCA2c</td>
<td>Testis</td>
</tr>
<tr>
<td></td>
<td>PMCA2w</td>
<td>Brain, kidney, uterus</td>
</tr>
<tr>
<td></td>
<td>PMCA2x</td>
<td>Brain, heart</td>
</tr>
<tr>
<td></td>
<td>PMCA2y</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PMCA2z</td>
<td>Brain, heart</td>
</tr>
<tr>
<td>PMCA3 (the human gene is located on Xq28)</td>
<td>PMCA3a</td>
<td>Brain, spinal cord, testis</td>
</tr>
<tr>
<td></td>
<td>PMCA3b</td>
<td>Adrenal, brain, skeletal muscle</td>
</tr>
<tr>
<td></td>
<td>PMCA3c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PMCA3d</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>PMCA3e</td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td></td>
<td>PMCA3f</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PMCA3x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PMCA3z</td>
<td></td>
</tr>
<tr>
<td>PMCA4 (the human gene is located on 1q25−q32)</td>
<td>PMCA4a</td>
<td>Widespread</td>
</tr>
<tr>
<td></td>
<td>PMCA4b</td>
<td>Ubiquitous; a housekeeper pump</td>
</tr>
<tr>
<td></td>
<td>PMCA4x</td>
<td>Widespread</td>
</tr>
<tr>
<td></td>
<td>PMCA4z</td>
<td>Heart, testis</td>
</tr>
<tr>
<td><strong>Sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERCA1</td>
<td>SERCA1a</td>
<td>Fast twitch skeletal muscle</td>
</tr>
<tr>
<td></td>
<td>SERCA1b</td>
<td>Fast twitch skeletal muscle</td>
</tr>
<tr>
<td>SERCA2</td>
<td>SERCA2a</td>
<td>Cardiac and slow twitch skeletal muscle</td>
</tr>
<tr>
<td></td>
<td>SERCA2b</td>
<td>Ubiquitous; a housekeeper pump in smooth muscle and many other cells</td>
</tr>
<tr>
<td></td>
<td>SERCA2c</td>
<td>Heart and skeletal muscle</td>
</tr>
<tr>
<td>SERCA3</td>
<td>SERCA3a</td>
<td>Mast cells, lymphocytes, platelets, monocytes, vascular endothelial cells and cerebellar Purkinje cells</td>
</tr>
<tr>
<td></td>
<td>SERCA3b</td>
<td>Haematopoietic cells; blood platelets</td>
</tr>
<tr>
<td></td>
<td>SERCA3c</td>
<td>Haematopoietic cells; Blood platelets</td>
</tr>
<tr>
<td></td>
<td>SERCA3d</td>
<td>Heart and skeletal muscle</td>
</tr>
<tr>
<td></td>
<td>SERCA3e</td>
<td>Pancreas and lung</td>
</tr>
<tr>
<td></td>
<td>SERCA3f</td>
<td>Heart and skeletal muscle</td>
</tr>
<tr>
<td><strong>Secretory-pathway Ca^{2+}-ATPase (SPCA) pumps</strong></td>
<td>SPCA1</td>
<td>Ubiquitous; located in the Golgi.</td>
</tr>
<tr>
<td></td>
<td>SPCA2</td>
<td></td>
</tr>
<tr>
<td><strong>Na^{+}/Ca^{2+} exchangers (NCXs)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCX1</td>
<td></td>
<td>Heart, kidney</td>
</tr>
<tr>
<td>NCX2</td>
<td></td>
<td>Neurons</td>
</tr>
<tr>
<td>NCX3</td>
<td></td>
<td>Neurons</td>
</tr>
<tr>
<td><strong>Na^{+}/Ca^{2+}/K^{+} exchangers (NCKXs)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCKX1</td>
<td>Rod photoreceptors (Module 10: Figure phototransduction) and platelets</td>
<td></td>
</tr>
<tr>
<td>NCKX2</td>
<td>Brain, cone photoreceptors</td>
<td></td>
</tr>
<tr>
<td>NCKX3</td>
<td>Brain, aorta, uterine and intestine</td>
<td></td>
</tr>
<tr>
<td>NCKX4</td>
<td>Brain, aorta, lung and thymus</td>
<td></td>
</tr>
</tbody>
</table>

With regard to the distribution, the list is not complete, but includes those tissues where the different isoforms are strongly expressed. For a detailed description of the nomenclature and distribution of spliced variants, see Strehler and Zacharias 2001 for the PMCA pumps and Schnetkamp 2004 for NCKX.

**ATP binding and the loading of Ca^{2+} on to the binding site**

The first steps in the pump cycle (Steps 1 and 2 in Module 5: Figure SERCA pump cycle) is ATP binding and the loading of Ca^{2+} on to the external binding sites. This ATP binds to the N domain, which forms a cap sitting over the P domain (Module 5: Figure SERCA1a pump). The resulting conformational change within the M (transmembrane) domains opens up two Ca^{2+}-binding sites. The first point to notice is that the N domain is somewhat removed from the transmembrane domains where Ca^{2+} translocates through the membrane. The transmission of such a conformational change has to be mediated through long-range allosteric interactions. The pathway for transmitting such molecular changes is still not clear, but there are several possibilities. One possibility is that the A domain (also referred to as the transducer domain) may play a role. Another possibility is that information might be transmitted through the long rod-like central helix of M5 that extends from the inside of the membrane right up to the underside of...
The sarco/endo-plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump cycle.

The pump cycle consists of a series of biochemical reactions during which the pump switches between two major conformational states: an \(E_1\) state when the Ca\(^{2+}\)-binding site faces the cytoplasm, and an \(E_2\) state where the binding sites have switched to the opposite side and Ca\(^{2+}\) is released to the lumen. During each cycle, two Ca\(^{2+}\) ions are pumped for each ATP hydrolysed. This switch between the \(E_1\) and \(E_2\) states represents one cycle, and this occurs at a frequency of about 150 reactions/s. The way in which ATP powers each pump cycle is described in the text.

Phosphorylation of the aspartate residue and Ca\(^{2+}\) translocation through the membrane

A critical phase in the transport process is the interaction between the N and P domains during which the terminal phosphate group of ATP is transferred to Asp-351. The problem here is that the ATP-binding site on the N domain appears from the structure to be located far away from this phosphorylation site. Somehow the two domains have to move in order for the two sites to approach close enough for the phosphorylation to occur. Once the energized \(E_1\)~\(\sim P\) state is formed, another conformational change transmitted through the mechanisms discussed earlier brings about the movement of the M domains so that the Ca\(^{2+}\)-binding pocket is altered to face the lumen to allow Ca\(^{2+}\) to enter the endoplasmic reticulum (ER). This remarkable molecular machine is beautifully designed to efficiently couple the site of energy conversion (the phosphorylation domain) to the translocation mechanism in the membrane.

Ca\(^{2+}\) pump regulation

One of the hallmarks of Ca\(^{2+}\) pumps is their regulation, which enables them to adapt to changing circumstances. The most direct form of regulation is for Ca\(^{2+}\) to regulate its own activity, and this is particularly apparent for the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) pump. Ca\(^{2+}\) acts through calmodulin (CaM) to stimulate the pump. When the pump is activated through the Ca\(^{2+}\)/CaM mechanism, the CaM remains bound for some time after Ca\(^{2+}\) signalling has ceased, thus allowing the pump to have a ‘memory’ so that it can respond more quickly to another Ca\(^{2+}\) transient.

The Ca\(^{2+}\) pumps are sensitive to hormonal regulation, with control being exerted through various regulators such as phospholamban (PLN) and sarcolipin (SLN). PLN is particularly important, as it is sensitive to various signalling pathways operating through second messengers such as cyclic AMP and Ca\(^{2+}\) itself. Such regulation is critical for regulating cardiac contractility (Module 7: Figure ventricular Ca\(^{2+}\) signalling), where the strength of contraction is controlled by cyclic AMP, which acts by phosphorylating PLN to remove its inhibitor effect on
Module 5: Figure SERCA1a pump

Functional operation of the sarco/endo-plasmic reticulum Ca\(^{2+}\)-ATPase 1a (SERCA1a) pump

The main domains of the sarco/endo-plasmic reticulum Ca\(^{2+}\)-ATPase 1a (SERCA1a) pump concerned with pumping are the nucleotide-binding domain (N), the phosphorylation domain (P), the actuator domain (A) and the transmembrane domains (M1–M10). The molecular events involving these domains are described in the text by reference to the transition states of the pump cycle. Reproduced by permission from Macmillan Publishers Ltd: Nature, MacLennan, D.H. and Green, N.M. (2000) Structural biology: pumping ions. 405:633–634. Copyright (2000); see MacLennan and Green 2000.

1. A PLN pentamer, which forms when five PLN monomers come together, is stabilized by leucine–isoleucine zipper interactions.
2. Monomeric unphosphorylated PLN is the active form that binds to SERCA2a.
3. PLN binds in a groove running up both the transmembrane and cytosolic regions of SERCA2a (Module 5: Figure SERCA pump structure). PLN exerts its inhibitory action by regulating the Ca\(^{2+}\) affinity of the SERCA2a pump.
4. The SERCA2a pump increases its Ca\(^{2+}\) pumping activity when the inhibitory effect of PLN is removed following its phosphorylation by protein kinase A (PKA) or Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII).
5. Inactive PLN is phosphorylated on either Ser-16 (by PKA) or Thr-17 (by CaMKII) (Module 5: Figure phospholamban and sarcoplasm).
6. The phosphorylated PLN is converted back into its active inhibitory form following dephosphorylation by protein phosphatase PP1 (Module 5: Figure PP1 targeting to glycogen).

Studies on transgenic mice have established that PLN features significantly in the relationship between Ca\(^{2+}\) signalling and cardiac hypertrophy. There are also two examples of inherited human dilated cardiomyopathy that have been traced to mutations in PLN.
Phospholamban (PLN) has two main regions: a C-terminal α-helix that embeds the molecule in the sarcoplasmic reticulum (SR) membrane and an N-terminal cytoplasmic region. The latter has an α-helical region that is connected to the membrane region by a short β-turn. Sarcolipin (SLN) resembles PLN, except that it lacks most of the cytoplasmic domain. The high degree of homology, both conserved residues (grey/green) and identical residues (red), within the transmembrane domain indicates that this region is of particular significance in mediating the ability of these proteins to interact with, and inhibit, SERCA pumps. This inhibitory effect of PLN is reversed by protein kinase A (PKA) and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) phosphorylating Ser-16 and Thr-17 respectively. Reproduced by permission from Macmillan Publishers Ltd: MacLennan, D.H. and Kranias, E.G. (2003) Phospholamban and sarcolipin: a crucial regulator of cardiac contractility. 4:566–577. Copyright (2003); http://www.nature.com/nrm; see MacLennan and Kranias 2003.

**Sarcolipin (SLN)**

Sarcolipin (SLN) resembles phosphorylamban (PLN) with regard to its transmembrane region, but its cytoplasmic region has been truncated (Module 5: Figure phosphorylamban and sarcolipin). SLN is strongly expressed in fast-twitch skeletal muscle, but is low in heart. Like PLN, SLN functions to regulate sarco/endo-plasmic reticulum Ca²⁺-ATPase (SERCA) activity and appears to bind to a similar groove in the SERCA molecule.

**Secretory-pathway Ca²⁺-ATPase (SPCA)**

The Golgi contains both a sarco/endo-plasmic reticulum Ca²⁺-ATPase (SERCA) pump and a secretory-pathway Ca²⁺-ATPase (SPCA) that is responsible for pumping Ca²⁺ into the Golgi stacks. There is a variable distribution of these two pumps within the Golgi. The SERCA is found in the early parts of the Golgi, thus reflecting its origin from the endoplasmic reticulum (ER). The SPCA is restricted to the trans-Golgi region. The SPCA pumps Ca²⁺ into the Golgi using mechanisms similar to those described above for the SERCA pumps. Unlike the SERCA pumps, however, the SPCA is able to pump Mn²⁺ equally as well as Ca²⁺.

There are two SPCA isoforms (SPCA1 and SPCA2). The ATP2C1 gene that encodes SPCA1 is mutated in Hailey-Hailey disease.

**Sodium/calcium exchangers (NCX and NCKX)**

The Na⁺/Ca²⁺ exchangers play a critical role in Ca²⁺ signalling because they provide a mechanism for rapidly extruding Ca²⁺ from cells (Module 5: Figure Ca²⁺ uptake and extrusion). These exchangers are particularly important in excitable cells such as cardiac cells, neurons and sensory neurons. However, they are also expressed on various non-excitable cells. There are two families of exchangers, the Na⁺/Ca²⁺ exchanger (NCX) and the Na⁺/Ca²⁺–K⁺ exchanger (NCKX) families (Module 5: Table Ca²⁺ pumping toolkit).

**Na⁺/Ca²⁺ exchanger (NCX)**

The Na⁺/Ca²⁺ exchanger (NCX) was the first exchanger to be discovered. It functions to extrude Ca²⁺ from the cell in exchange for Na⁺ (Module 5: Figure sodium/calcium exchangers). The energy from the Na⁺ gradient across the plasma membrane is used to drive Ca²⁺ out of the cell against the large electrochemical gradient. There is no direct role for ATP, but it does have an indirect role because it powers the ouabain-sensitive Na⁺ pump that establishes the Na⁺ gradient. In kidney tubule cells, the NCX plays an important role in the reabsorption of Ca²⁺ by the paracellular transport pathway (Module 7: Figure kidney Ca²⁺ reabsorption).

The NCX has nine transmembrane (TM) segments with a large cytoplasmic loop connecting TM5 and TM6. The regions shaded in yellow in Module 5: Figure so- dium/calcium exchangers contain α repeats (α1 and α2), which are highly homologous with two similar regions in the Na⁺/Ca²⁺–K⁺ exchanger (NCKX). These are the only two regions that show close homology, and this would fit with the notion that α1 and α2 play a role in the binding and transport of cations.

NCX can operate in two modes, depending on the electrochemical potential, which determines the directionality of the Na⁺ flux that drives the movement of Ca²⁺. In the forward mode, Ca²⁺ is extruded from the cell, whereas in the reverse mode, Ca²⁺ is brought into the cell. This reverse mode may play an important role during excitation–contraction (E-C) coupling in heart cells, where the rapid build-up of Na⁺ during the course of the action potential results in a local build-up of this ion, which then begins to flow out in exchange for Ca²⁺. This influx of Ca²⁺ will contribute to the trigger Ca²⁺ entering through the L-type channel, and thus could facilitate the excitation processes. The NCX1 isoform has an important role in ventricular cell Ca²⁺ signalling (Module 7: Figure ventricular Ca²⁺ signalling).
Phospholamban (PLN) operates within the plane of the sarcoplasmic reticulum (SR) membrane to regulate the activity of the sarco/endo-plasmic reticulum Ca\(^{2+}\)-ATPase 2a (SERCA2a) pump. PLN has five distinct functional states, as described in the text.

There is some debate concerning the exact stoichiometry of NCX. Most measurements suggest that three Na\(^+\) ions are transported for each Ca\(^{2+}\) ion, which means that the exchanger is electrogenic.

**Na\(^+\)/Ca\(^{2+}\)–K\(^+\) exchanger (NCKX)**

The Na\(^+\)/Ca\(^{2+}\)–K\(^+\) exchangers (NCKXs) were first discovered in rod photoreceptors. There is a family of these exchangers with different distributions in both excitable and non-excitable cells (Module 5: Table Ca\(^{2+}\) pumping toolkit). They differ from the Na\(^+\)/Ca\(^{2+}\) exchangers (NCXs) in that they extrude both Ca\(^{2+}\) and K\(^+\) in exchange for Na\(^+\) (Module 5: Figure sodium/calcium exchangers). While some of their structural features resemble those found in NCX, there clearly are marked differences. The regions of closest homology are the two regions of \(\alpha\) repeats (\(\alpha1\) and \(\alpha2\) shown in yellow), which play a role in binding cations during the exchange reaction.

The function of these exchangers has been defined best in photoreceptors, where they are the primary mechanism for extruding Ca\(^{2+}\) during the process of phototransduction (see Step 5 in Module 10: Figure phototransduction overview). The organization of the NCKX1 isoform in the photoreceptor is of interest because it appears to be complexed to the cyclic nucleotide-gated channel (CNGC) that is responsible for the cyclic GMP-dependent entry of Ca\(^{2+}\). Furthermore, this entry channel/exchanger complex also appears to be linked to two proteins (peripherin and Rom-1) located in the rim of the intracellular disc. The functional significance of linking the plasma membrane to the internal disc through this protein complex is unknown.

**Mitochondria**

Mitochondria distributed throughout the cytoplasm have many functions. They generate ATP, they shape Ca\(^{2+}\) signals and respond to Ca\(^{2+}\) signals by increasing the production of ATP, they generate reactive oxygen species (ROS), and under extreme conditions, they release factors such as cytochrome c to induce apoptosis. Their primary function is the generation of ATP by oxidative phosphorylation. In addition, they also play a critical role in a number of other aspects of cell signalling, particularly Ca\(^{2+}\) signalling. Mitochondria contribute to the dynamics of Ca\(^{2+}\) signalling (Module 5: Figure Ca\(^{2+}\) uptake and extrusion) by participating in the OFF reactions that remove Ca\(^{2+}\) from the cytoplasm during the recovery phase (Module 2: Figure Ca\(^{2+}\) transient mechanisms). The mitochondrial Ca\(^{2+}\) uptake process is responsible for the mitochondrial modulation of Ca\(^{2+}\) signals. Mitochondria function as Ca\(^{2+}\) buffers capable of shaping both the amplitude and the spatiotemporal profile of Ca\(^{2+}\) signals. Mitochondrial Ca\(^{2+}\) release mechanisms return Ca\(^{2+}\) back into the cytosol, where it can be sequestered by the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR). Indeed, there is a close functional relationship between the mitochondria and the ER/SR.
An endoplasmic reticulum (ER)/mitochondrial Ca\textsuperscript{2+} shuttle, which is important for intracellular Ca\textsuperscript{2+} dynamics and cell signalling, can be both beneficial and deleterious. With regard to the former, an increased Ca\textsuperscript{2+} concentration within the mitochondrial matrix stimulates enzymes associated with the tricarboxylic acid (TCA) cycle, resulting in an increase in ATP production. Therefore there is a two-way relationship between cytosolic Ca\textsuperscript{2+} signals and mitochondrial function. In addition to the mitochondrial modulation of Ca\textsuperscript{2+} signals, mentioned above, there is a reciprocal Ca\textsuperscript{2+} modulation of mitochondrial function. For example, the uptake of Ca\textsuperscript{2+} acts to stimulate the oxidative processes that produce ATP. This increase in oxidation also enhances mitochondrial reactive oxygen species (ROS) formation (Module 2: Figure sites of ROS formation) that contributes to the redox signalling pathway. In addition, an alteration in the normal eb and flow of Ca\textsuperscript{2+} through the mitochondria can be deleterious when an abnormally high load of Ca\textsuperscript{2+} is transferred from the ER/SR to the mitochondrion. This excessive uptake of Ca\textsuperscript{2+} into the mitochondria can activate the formation of the mitochondrial permeability transition pore (MTP), which results in the release of proteins such as cytochrome c, which induce the caspase cascade that contributes to the apoptotic signalling network. Indeed, a large number of cell death signals appear to operate through the ER/mitochondrial Ca\textsuperscript{2+} shuttle.

**Generation of ATP**

The mitochondrion is often referred to as the ‘powerhouse’ of the cell because of its ability to generate ATP. However, this role in energy transformation is intimately connected with its other role, which is to modulate Ca\textsuperscript{2+} signalling (Module 5: Figure mitochondrial Ca\textsuperscript{2+} signalling). Mitochondria are powered by pyruvate, which enters the tricarboxylic acid (TCA) cycle to produce NADH, which provides the electrons that travel down the electron transport chain, during which protons are ejected into the cytoplasm. Oxygen is the final electron acceptor, but approximately 25% of the oxygen consumed is incompletely reduced and appears as the superoxide radical (O\textsubscript{2}•−), and this mitochondrial reactive oxygen species (ROS) formation can contribute to the redox signalling pathway. The removal of H\textsuperscript{+} creates the large membrane potential of approximately 250 mV, which is used to energize both ATP synthesis by the ATP synthase and the uptake of Ca\textsuperscript{2+} by the uniporter. This uptake of Ca\textsuperscript{2+} has a maximum velocity that is very much larger than the Ca\textsuperscript{2+} exchanger that returns Ca\textsuperscript{2+} to the cytoplasm, which means that mitochondria can rapidly accumulate large amounts of Ca\textsuperscript{2+}, much of which is bound to mitochondrial buffers or it precipitates as crystals of calcium phosphate. These buffers ensure that the concentration of Ca\textsuperscript{2+} within the matrix does not rise much above 1 μM. During the recovery phase of Ca\textsuperscript{2+} signals, the accumulated Ca\textsuperscript{2+} is returned to the cytoplasm by the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Under exceptional circumstances, when Ca\textsuperscript{2+} overwhelms the mitochondria, the mitochondrial permeability transition pore (MTP) is activated to speed up the release of Ca\textsuperscript{2+}. Ca\textsuperscript{2+} has two main signalling functions within the mitochondrial matrix: it activates the MTP and it also stimulates the TCA cycle to enhance the formation of ATP. One of the consequences of the latter is an increase in the production of O\textsubscript{2}•−, which acts synergistically with Ca\textsuperscript{2+} to activate the MTP.

**Mitochondrial Ca\textsuperscript{2+} uptake**

In resting cells, the concentration of Ca\textsuperscript{2+} within the mitochondrial matrix is 80–200 nM, which is close to the level in the cytoplasm. When the cytosolic level of Ca\textsuperscript{2+} begins to rise, Ca\textsuperscript{2+} enters the mitochondrion through a Ca\textsuperscript{2+} mitochondrial uniporter driven by the same proton gradient that is used to power ATP synthesis (Module 5: Figure mitochondrial Ca\textsuperscript{2+} signalling). A mitochondrial Ca\textsuperscript{2+} release mechanism then returns Ca\textsuperscript{2+} from the mitochondrial matrix back into the cytoplasm. This ebb and flow of Ca\textsuperscript{2+} through the mitochondrion is dramatically demonstrated when cells are generating repetitive Ca\textsuperscript{2+} spikes (Module 5: Figure mitochondrial Ca\textsuperscript{2+} oscillations).

**Mitochondrial uniporter**

A uniporter located in the inner mitochondrial membrane is responsible for taking up Ca\textsuperscript{2+} from the cytoplasm (Module 5: Figure mitochondrial Ca\textsuperscript{2+} signalling). Since...
Module 5: Figure sodium/calcium exchangers

Structure and function of sodium/calcium exchangers.
The organization and operation of the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) is shown on the left and the Na\(^+\)/Ca\(^{2+}\)–K\(^+\) exchanger (NCKX) is shown on the right. There is a large central cytoplasmic loop, part of which has a site where alternative splicing occurs. NCX also has a Ca\(^{2+}\) regulatory site and a binding site for the exchanger inhibitory peptide (XIP). Both exchangers have two regions (\(\alpha_1\) and \(\alpha_2\), shown in yellow) containing \(\alpha\) repeats that are highly homologous and are thought to be the sites where the cations bind to the exchangers. See the text for further details.

Module 5: Figure mitochondrial Ca\(^{2+}\) signalling

Relationship between mitochondrial metabolism and Ca\(^{2+}\) signalling
Mitochondria are energized by pyruvate, which enters the mitochondrion where it is metabolized by the tricarboxylic acid (TCA) cycle (or citric acid cycle) to produce the reducing equivalents that are transferred to the electron transport chain located in the inner mitochondrial membrane. The subsequent extrusion of protons creates a large (\(-150\) to \(-180\) mV) negative membrane potential across this inner membrane. This large proton gradient is used to energize both ATP synthesis and the uptake of Ca\(^{2+}\). Protons flowing back through the oligomycin-sensitive ATP synthase drive the synthesis of ATP.
Mitochondrial tracking of cytosolic Ca\textsuperscript{2+} transients in hepatocytes.


the uniporter functions as a channel, it is able to take up Ca\textsuperscript{2+} over a wide range of concentrations. It can take up Ca\textsuperscript{2+} slowly at the normal global levels of Ca\textsuperscript{2+} (approximately 500 nM). As the concentration continues to rise, uptake increases in a steeply Ca\textsuperscript{2+} concentration-dependent manner, with half-maximal activation occurring at around 15 µM. Mitochondria can accumulate as much as 25–50% of the Ca\textsuperscript{2+} released from the endoplasmic reticulum (ER).

Mitochondrial Ca\textsuperscript{2+} release

The Ca\textsuperscript{2+} that is taken up by mitochondria during signalling is released back to the cytoplasm by various efflux pathways:

Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} efflux

Like so many functions in the mitochondrion, this mode of efflux is driven by the negative membrane potential. Ca\textsuperscript{2+} is extruded from the mitochondrion by means of a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, which has a stoichiometry of three Na\textsuperscript{+} ions for one Ca\textsuperscript{2+} ion. The Na\textsuperscript{+} that enters down the electrochemical gradient is exchanged for Ca\textsuperscript{2+} (Module 5: Figure mitochondrial Ca\textsuperscript{2+} signalling). Pharmacological agents such as amiloride, diltiazem, bepridil and CGP37157 inhibit the exchanger.

Na\textsuperscript{+}-independent Ca\textsuperscript{2+} efflux

Mitochondria appear to have a Na\textsuperscript{+}-independent Ca\textsuperscript{2+} release mechanism that is rather slow and may play a role in extruding Ca\textsuperscript{2+} under resting conditions. Extrusion is dependent on the transmembrane potential, and may depend upon an H\textsuperscript{+}/Ca\textsuperscript{2+} antiporter.

Endoplasmic reticulum (ER)/mitochondrial Ca\textsuperscript{2+} shuttle

The function of the endoplasmic reticulum (ER) is intimately connected with that of the mitochondria. The concept of an ER/mitochondrial Ca\textsuperscript{2+} shuttle has emerged from the fact that the ER and the mitochondria form a highly dynamic interconnected network that functions both to generate and to modulate Ca\textsuperscript{2+} signals (Module 5: Figure ER/mitochondrial shuttle). The close association between these two organelles is maintained by the mitofusins (MFNs). The Ca\textsuperscript{2+} stored within the ER lumen is released into the cytoplasm by inositol 1,4,5-trisphosphate receptors (InsP\textsubscript{3}Rs) and ryanodine receptors (RYRs) to provide the cytosolic Ca\textsuperscript{2+} signal to activate many cellular processes. During the recovery phase, this cytosolic Ca\textsuperscript{2+} can be dealt with in different ways. It can be returned directly to the ER by the sarco/endo-plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) pump. Alternatively, Ca\textsuperscript{2+} is taken up by the mitochondrion and then returned to the ER through the ER/mitochondrial shuttle. The mitochondria assist with recovery phase by rapidly sequestering some of the released Ca\textsuperscript{2+} and then later returning it to the ER. During normal signalling there is therefore a continuous ebb and flow of Ca\textsuperscript{2+} between these two organelles. The normal situation is for most of the Ca\textsuperscript{2+} to reside within the lumen of the ER, except during Ca\textsuperscript{2+} signalling, when a variable proportion passes through the
The endoplasmic reticulum (ER)/mitochondrial Ca\(^{2+}\) shuttle.

The endoplasmic reticulum (ER) and the mitochondria are functionally linked through their participation in Ca\(^{2+}\) signalling. During Ca\(^{2+}\) signalling, a small bolus of Ca\(^{2+}\) is periodically released to the cytoplasm by the inositol 1,4,5-trisphosphate receptors (InsP\(_3\)R). While most of the Ca\(^{2+}\) is returned to the ER by the sarco/endo-plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump, a proportion enters the mitochondria through the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (OMM), and then through the uniporter in the inner mitochondrial membrane (IMM). The Ca\(^{2+}\) in the mitochondrial matrix is then returned to the ER through a series of exchangers, channels and pumps. Firstly, Ca\(^{2+}\) leaves the matrix through a Na\(^{+}\)/Ca\(^{2+}\) exchanger and then enters the cytoplasm through the VDAC. Once in the cytoplasm, it can be taken back into the ER by the SERCA pumps.

Mitochondrial permeability transition pore (MTP)

When describing mitochondrial Ca\(^{2+}\) release mechanisms, it is important to include the mitochondrial permeability transition pore (MTP), which is particularly important with regard to the activation of apoptosis. The MTP is sometimes referred to as the permeability transition pore (PTP). The MTP is the focal point of many apoptotic signals, including Ca\(^{2+}\), reactive oxygen species (ROS) and possibly also members of the Bcl-2 superfamily. One of the signals activating the MTP is an abnormal accumulation of Ca\(^{2+}\) in the mitochondria, which results in collapse of the mitochondrial membrane potential and the release of factors that activate the caspase cascade (Module 5: Figure ER/mitochondrial shuttle). Mitochondria thus play a pivotal role in regulating apoptosis because they lie at the centre of a complex web of interactions that link apoptotic signals to the caspase cascade (Module 11: Figure apoptosis).

The MTP is a non-selective channel with a very high conductance (pore radius 1–1.3 nm) capable of releasing both metabolites and ions. The sudden release of protons completely depolarizes the potential with immediate cessation of most mitochondrial functions, together with a catastrophic release of essential components such as cytochrome c and apoptosis-inducing factors (AIFs) that then go on to activate apoptosis (Module 5: Figure ER/mitochondrial shuttle). As such, the mitochondrion has a central role in the process of apoptosis, and there is
much interest concerning the nature of this channel and how it is activated.

The MTP is composed of components that have normal functions within the mitochondrion. The pore in the inner mitochondrial membrane (IMM) is the adenine nucleotide translocase (ANT) that normally functions as a gated port mediating the entry of ADP and the release of ATP. Under certain conditions, especially a high level of Ca\(^{2+}\) within the matrix, the translocase opens up to form the non-selective pore. Bongkrekic acid, which binds to ANT, is a potent inhibitor of apoptosis. This ANT is associated with the voltage-dependent anion channel (VDAC), which normally functions to enhance the permeability of the outer membrane. Pore opening requires cyclophilin-D (CyP-D), which might act to control the assembly of the MTP. CyP-D is a mitochondrial isoform of a family of cyclophilins that are sensitive to cyclosporin A (CsA), which not only functions as an immunosuppressant, but also is a potent inhibitor of apoptosis.

A large number of factors control the opening of the MTP. Two key factors are an increase in matrix Ca\(^{2+}\) concentration and oxidation of diithiols located on ANT. Opening of the MTP seems to require both oxidative stress and an increase in Ca\(^{2+}\) (Module 5: Figure mitochondrial Ca\(^{2+}\) signalling). It seems that overloading of the mitochondrion is not in itself deleterious, unless it occurs in the presence of other factors, such as a change in the redox state or a decline in the level of ATP. Some of the free radicals that open MTP are produced within the mitochondrion, because some of the oxygen is incompletely reduced and appears as the superoxide radical (O\(_2^-\)). The O\(_2^-\) can then oxidize the vic-thiols on ANT to facilitate the conformational change that opens the pore (Module 5: Figure mitochondrial Ca\(^{2+}\) signalling). This oxidative mechanism is normally prevented by the highly reduced state within the mitochondrial matrix that is maintained by high levels of glutathione (GSH).

**Mitochondrial modulation of Ca\(^{2+}\) signals**

Mitochondria can take up large quantities of Ca\(^{2+}\) very rapidly and thus can modulate various aspects of Ca\(^{2+}\) signalling. In addition to functioning as a Ca\(^{2+}\) buffer, mitochondria can also modulate the flow of Ca\(^{2+}\) through both the entry and release channels.

Mitochondria function as immobile buffers. The Ca\(^{2+}\) that is taken up during the course of a Ca\(^{2+}\) signal is then released back into the cytoplasm, where it is either returned to the endoplasmic reticulum (ER) or pumped out of the cell. The level of Ca\(^{2+}\) within the mitochondrial matrix is held constant by means of buffers and by formation of a calcium phosphate precipitate (Module 5: Figure mitochondrial Ca\(^{2+}\) signalling). During prolonged periods of stimulation, large amounts of Ca\(^{2+}\) are taken up by the mitochondrion, and this is then gradually unloaded during periods of rest. In the case of nerve terminals, for example, it can take up to 10 min for the mitochondrial level of Ca\(^{2+}\) to return to its resting level following a period of intense stimulation.

This ability of Ca\(^{2+}\) to sequester large amounts of Ca\(^{2+}\) can markedly modify both the shape and the amplitude of cytosolic Ca\(^{2+}\) signals. An example of the former is the ability of mitochondria to enhance Ca\(^{2+}\) signals by dampening out the negative feedback effects that normally limit the activity of Ca\(^{2+}\) channels, as occurs in T cells (Module 9: Figure T cell Ca\(^{2+}\) signalling). When mitochondria are inhibited, the entry of external Ca\(^{2+}\) is markedly reduced (Module 5: Figure mitochondria and Ca\(^{2+}\) entry).
Mitochondria can also modify the shape of Ca\(^{2+}\) transients, which depend upon the sequential activation of ON and OFF reactions (Module 2: Figure Ca\(^{2+}\) transient mechanisms). The sharpness of the transients depends not only on how quickly Ca\(^{2+}\) is introduced into the cytoplasm, but also on how quickly it is removed by the various OFF reactions. The mitochondria play an important role in the kinetics of the recovery phase because this becomes considerably prolonged when their activity is inhibited (Module 5: Figure chromaffin cell Ca\(^{2+}\) transients).

Ca\(^{2+}\) modulation of mitochondrial function

Aerobic ATP synthesis by the mitochondria is tightly regulated. There is a direct control mechanism exercised through the ATP/ADP ratio that automatically increases metabolism when the level of ATP declines. In addition, Ca\(^{2+}\) functions as a catabolic signal in that it activates pyruvate dehydrogenase, oxoglutarate dehydrogenase and isocitrate dehydrogenase, key regulatory enzymes of the tricarboxylic acid (TCA) cycle (Module 5: Figure mitochondrial Ca\(^{2+}\) signalling). When Ca\(^{2+}\) builds up within the mitochondrion, it activates the TCA cycle, that then increases the supply of reducing equivalents and hence an increase in ATP formation (Module 5: Figure cytosolic and mitochondrial Ca\(^{2+}\) transients). This feedback mechanism is an example of the interaction between metabolic messengers and cell signalling pathways (Module 2: Figure metabolic signalling). Such an interaction may explain how pyruvate can markedly enhance cardiac cell Ca\(^{2+}\) signalling (Module 2: Figure pyruvate and Ca\(^{2+}\) signalling).

The increase in mitochondrial metabolism will also enhance the formation of the superoxide radical (O\(_2^{-}\)), and this mitochondrial reactive oxygen species (ROS) formation can contribute to redox signalling. In addition, O\(_2^{-}\) formation within the mitochondria can act synergistically
with Ca\(^{2+}\) to open the mitochondrial permeability transition pore (MTP).

**Mitochondrial motility**

Mitochondria are not static in cells. They move around to cellular regions where metabolic demands are high. This movement is particularly evident in neurons, where their energy demands are widely dispersed because of their complex morphology (Module 10: Figure neuronal morphology). For example, during gene transcription, energy is required at the soma, but when information is being processed at the synapses on the spines and dendrites, energy demand will shift from the cell body to the periphery.

Mitochondria travel around the cell attached to the microtubules (MTs) and are propelled by plus end-directed kinesin and minus end-directed dynein motor proteins (Module 5: Figure mitochondrial motility). The way in which these motors are controlled to direct mitochondria to different regions in the cell is still somewhat of a mystery. However, there is clear experimental evidence that the movement of mitochondria is rapidly inhibited by increases in intracellular Ca\(^{2+}\). One way in which Ca\(^{2+}\) inhibits mitochondrial movement depends on the mitochondrial Rho-GTPase (Miro) protein family (Miro 1 and Miro 2). These Rho-GTPases have two EF-hand Ca\(^{2+}\)-binding domains. A current hypothesis is that Miro and associated proteins, such as Milton, might be part of a complex that attaches the mitochondria to the kinesin motor, which is the primary anterograde mitochondrial motor. In regions of high Ca\(^{2+}\), Miro functions as the sensor responsible for detecting Ca\(^{2+}\) and this results in the motor detaching from the microtubule (Module 5: Figure mitochondrial motility). The motor domain of kinesin interacts with Miro when the latter is bound to Ca\(^{2+}\). Such a mechanism could explain how mitochondria accumulate in regions where there is intense activity, since this is also likely to be where there are microdomains of Ca\(^{2+}\).

**Mitochondrial Rho-GTPase (Miro)**

The mitochondrial Rho-GTPase (Miro) protein family has two members (Miro 1 and Miro 2). They are GTPase-activating proteins (GAPs) that have two EF-hand Ca\(^{2+}\)-binding domains. They function together with Milton to form a complex that attaches the mitochondria to the kinesin motor (Module 5: Figure mitochondrial motility).

**Mitofusins (MFNs)**

The mitofusins (MFNs) are dynamin-like GTPases that function in mitochondrial fusion. They also have an additional function in holding together the mitochondria and the endoplasmic reticulum, which is of critical importance for the operation of the endoplasmic reticulum (ER)/mitochondrial Ca\(^{2+}\) shuttle (Module 5: Figure ER/mitochondrial shuttle). The ER membrane has MFN2 that forms dimeric anti-parallel complexes with either MFN2 or MFN1 located in the outer membrane of the mitochondrion.

Charcot-Marie-Tooth disease 2A is caused by mutations in MFN2.
References

Protein tyrosine phosphatases (PTPs)

Protein serine/threonine phosphatases

Phosphodiesterase (PDE)

Ca\textsuperscript{2+} pumps


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